Universidade de Lisboa
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COUPLING OF ATHEROSCLEROSIS PROGRESSION AND BONE DISTURBANCES IN INFLAMMATORY RHEUMATIC DISEASES

Diana de Almeida Carmona Fernandes

Orientador(es): Prof. Doutor João Eurico Cortez Cabral da Fonseca
Prof. Doutora Helena Cristina de Matos Canhão

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LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
ALP	Alkaline phosphatase
AIx	Augmentation index
AMI	Acute myocardial infarction
Anti-dsDNA	Antibodies against double-stranded DNA
Anti-Sm	Antibodies against Smith antigen
ApoE	Apolipoprotein E
BILAG	British Isles Lupus Assessment Group
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenic protein
BMU	Basic multicellular unit
BSP	Bone sialoprotein
CALCR	Calcitonin receptor
Cbfa1	Core-binding factor subunit alpha-1
cIMT	Carotid intima-media thickness
CLCN	Chloride channel
CRP	C-reactive protein
CSF1R	Colony stimulating factor 1 receptor
CTSK	Cathepsin K
CTX	Carboxy-terminal cross-linked telopeptide of type I collagen
CV	Cardiovascular
DAS28	Disease activity score 28 joints
DC	Dendritic cell
DKK1	Dickkopf-related protein 1
DXA	Dual x-ray absorptiometry
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cell
ESR	Erythrocyte sedimentation rate

EULAR	European league against rheumatism
FRAX®	Fracture risk assessment tool
FZ	Frizzled
GSK-3β	Glycogen synthase kinase-3β
HAQ-DI	Health assessment questionnaire disability index
HDL	High-density lipoprotein
HSC	Hematopoietic stem cell
ICAM-1	Intracellular adhesion molecule-1
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IgM	Immunoglobulin M
IL	Interleukin
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LEF	Lymphoid enhancer factor
LRP	Low-density lipoprotein receptor-related protein
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
MGP	Matrix gla protein
MSC	Mesenchymal stem cell
NFATc1	Nuclear factor of activated T cell c1
NF-ƙB	Nuclear transcription factor-ƙB
NTX	Amino-terminal cross-linked telopeptide of type I collagen
OB	Osteoblast
OC	Osteoclast
OCL	Osteocalcin
OP	Osteoporosis
OPG	Osteoprotegerin
OPN	Osteopontin
Osx	Osterix
P1CP	Carboxy-terminal propeptide of type I collagen
P1NP	Amino-terminal propeptide of type I collagen
PAT	Peripheral artery tonometry
RA	Rheumatoid arthritis

RANK	Receptor activator of nuclear factor-ƙB
RANKL	RANK ligand
RF	Rheumatoid factor
Runx2	Runt-related transcription factor 2
sFRP	Secreted Frizzled related protein
SLE	Systemic lupus erythematosus
SLEDAI	SLE disease activity index
SLICC-DI	Systemic Lupus International Collaborating Clinics / ACR Damage Index
SMC	Smooth muscle cell
SOST	Sclerostin
sRANKL	Soluble RANKL
TACE	TNF-α converting enzyme
TCF	T cell factor
TF	Tissue factor
TGF-β	Transforming growth factor beta
Th	T helper
TM	Thrombomodulin
TNF	Tumor necrosis factor
TNFR1	TNF receptor I
TNFR2	TNF receptor II
TRAF	TNF receptor-associated factor
TRAP	Tartrate-resistant acid phosphatase
VCAM-1	Vascular cell adhesion molecule-1
VE-cadherin	Vascular endothelial cadherin
VLDL	Very-low-density lipoprotein
VSMC	Vascular smooth muscle cell
WHO	World Health Organization
WIF	Wnt inhibitory factor
Wnt	Wingless-related integration site

RESUMO

As doenças cardiovasculares (CV) e a osteoporose (OP) estão entre os problemas de saúde mais comuns, contribuindo significativamente para o aumento da morbilidade e mortalidade da população. Segundo a Organização Mundial de Saúde, as doenças CV são a primeira causa de morte no mundo, e a aterosclerose é a principal causa de eventos CV. A OP é a doença óssea mais comum, caracterizada pela diminuição da densidade mineral óssea e aumento do risco de fraturas de fragilidade e da deterioração estrutural do osso. A incidência destas doenças aumenta com a idade e frequentemente coexistem no mesmo doente. Estudos epidemiológicos demonstraram claramente que, em indivíduos com patologia CV, o risco de fratura osteoporótica é 1,2 a 6,7 vezes maior do que na população geral. Essa relação também é observada em doentes com baixa massa óssea que apresentam um risco aumentado de eventos CV, calcificação vascular e progressão da aterosclerose.

A associação entre sistema vascular e esquelético ganhou destaque ao longo dos anos, especialmente devido ao paradoxo entre a calcificação vascular e a baixa densidade mineral óssea, sustentado por diversos estudos em ratinhos e humanos.

A calcificação vascular é um marcador do aumento do risco CV e tem sido apontada como um possível preditor de eventos CV. Acreditava-se que fosse um processo passivo, inevitável e natural do envelhecimento, mas essa deposição de minerais nos vasos mostrou-se mais complexa, ocorrendo de maneira organizada, não apenas como uma mera calcificação, mas como um processo semelhante à ossificação, com deposição de cristais de hidroxiapatite produzidos por células vasculares que têm a capacidade de mimetizar a diferenciação osteoblástica, formando uma estrutura semelhante ao osso no sistema vascular. Paralelamente, ocorre expressão de várias proteínas associadas ao desenvolvimento normal do osso, entre as quais OPG (osteoprotegerina), RANKL (receptor activador do factor nuclear ƙB), OPN (osteopontina), colagénio do tipo I e também citocinas pró-inflamatórias implicadas na modulação da imunidade adaptativa e consideradas potenciais reguladoras do desenvolvimento de lesões ateroscleróticas e calcificações. Apesar do sistema RANK/RANKL/OPG e da via Wingless (Wnt) serem característicos do metabolismo ósseo, estes foram identificados no desenvolvimento da aterosclerose e podem contribuir para a regulação da calcificação vascular. Além disso, o RANKL e a OPG são expressos não apenas por células relacionadas com o osso, mas também por muitos componentes do sistema CV, como as células endoteliais, as plaquetas e as células musculares lisas presentes nos vasos. Embora haja

uma forte associação entre estas proteínas e lesões ateroscleróticas, o mecanismo pelo qual o processo de calcificação é regulado ainda é desconhecido.
A associação entre as doenças CV e a OP é particularmente relevante em doentes com doenças inflamatórias crónicas, imunomediadas, como Lúpus Eritematoso Sistémico (LES) e Artrite Reumatoide (AR). As doenças reumáticas imunomediadas são distúrbios inflamatórios que envolvem vários órgãos e que têm como principais comorbilidades a doença CV e a OP. Estes doentes apresentam taxas aumentadas de aterosclerose subclínica, com um risco 5-8 e 2-3 vezes maior de ocorrência de eventos CV no LES e na AR, respectivamente, e um aumento da prevalência de OP em torno de 5-25% no LES e 15-35% na AR.

Este trabalho tem por hipótese que os distúrbios ósseos e vasculares partilham mecanismos fisiopatológicos comuns e que existe uma relação entre a progressão de ambas as condições. Assim, esta tese teve como objetivo compreender a interação entre os vasos e os ossos no contexto de doenças reumáticas inflamatórias através do estudo do efeito da inflamação nos tecidos.

Na primeira parte deste estudo avaliámos como, nas doenças reumáticas inflamatórias crónicas, especificamente AR e LES, os vasos estão modificados, levando a alterações vasculares e à doença CV. Para tal avaliámos as alterações vasculares precoces observadas nesses doentes sem eventos CV prévios, através da determinação dos níveis séricos de biomarcadores vasculares e do estudo da função endotelial. Após 5 anos, avaliámos a incidência de eventos CV na coorte de doentes com AR, e verificámos o contributo de factores de risco de doença CV tradicionais assim como de parâmetros relacionados com a AR para a ocorrência de eventos CV. Mostrámos que a AR e o LES apresentam padrões distintos nas alterações vasculares precoces, mais pronunciadas nos doentes com LES, e que a atividade inflamatória foi muito relevante para o aumento dos biomarcadores da ativação endotelial e para a disfunção endotelial. Além disso, os doentes com AR têm uma incidência de eventos CV muito maior do que a incidência relatada para a população geral Portuguesa, para os quais os marcadores inflamatórios e de ativação endotelial contribuem significativamente.

Na coorte de doentes com LES, avaliámos como os biomarcadores do metabolismo ósseo, especificamente OPG e RANKL, são afetados pela doença. Constatámos que esses doentes têm uma relação sRANKL/OPG aumentada, devido à redução dos níveis de OPG. Além disso, os níveis de OPG e sRANKL estão relacionados com os anticorpos específicos do LES, mas não com

terapêutica com corticosteroides, sugerindo que o aumento dos estímulos osteoclásticos é impulsionado pelos mecanismos próprios da doença.

Finalmente pretendemos compreender o papel da inflamação como denominador comum para a interação entre os ossos e os vasos. Para isso, usámos primeiramente um modelo de aterosclerose em ratinhos ($ApoE^{-/-}$) e avaliámos os distúrbios dos ossos e dos vasos e verificámos a sua associação com marcadores inflamatórios. Os nossos resultados sugerem que a inflamação não é o principal fator para a progressão da aterosclerose e para os distúrbios ósseos neste modelo animal, mas que as alterações no transporte lipídico e a dieta rica em gordura, associada à idade dos animais, podem desempenhar um papel mais expressivo.

Em segundo lugar, utilizando amostras de osso e de aorta de dadores de órgãos e placas de ateroma de doentes com aterosclerose avançada submetidos a endarterectomia carotídea, avaliámos o papel da inflamação na relação entre alterações ósseas e a patologia vascular. Os nossos resultados sugerem que a relação entre as alterações observadas nos ossos e nos vasos em contexto da doença aterosclerótica e da osteoporose podem depender de uma relação intrínseca entre os tecidos envolvidos, independentemente da progressão de ambas as doenças.

Os resultados obtidos sugerem que a relação entre a aterosclerose e a osteoporose depende de uma conexão intrínseca entre os tecidos e que a contribuição dos fatores de risco para a progressão da doença, incluindo a inflamação, afeta mais acentuadamente os doentes com doenças inflamatórias crónicas imunomediadas.

Palavras-chave: Aterosclerose, Osteoporose, Inflamação, Doenças Sistémicas Imunomediadas, Doenças Cardiovasculares.

ABSTRACT

Cardiovascular diseases (CV) and osteoporosis (OP) are among the most prevalent health problems, significantly contributing to increased morbidity and mortality of the population. The incidence of these conditions increases with age and they frequently coexist in the same patient. Epidemiological studies have demonstrated that patients with CV diseases have an increased risk for osteoporotic fractures and that patients with low bone mass have an increased risk for CV events. The inflammatory process has been implicated in the pathogenesis of both diseases, particularly in patients with immune-mediated rheumatic diseases like Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE).

In this work we hypothesize that bone and vascular disturbances share common pathophysiological mechanisms and that a connection between the progression of both conditions exists. Therefore, the aim of this thesis was to understand the interaction between vessels and bones in the context of inflammatory rheumatic diseases through the study of the effect of inflammation on tissues.

In the first part of this study we evaluated how, in chronic inflammatory rheumatic diseases, namely RA and SLE, the vessels are disturbed, leading to vascular alterations and CV disease. For that we have assessed the early vascular alterations observed in these patients without previous CV events, through the determination of serum levels of vascular biomarkers and the study of endothelial function. We have then assessed the incidence of CV events in the RA cohort after 5 years and identified the contribution of traditional CV disease risk factors and RA-related parameters to CV events. We found that RA and SLE show distinct patterns of early vascular changes, more pronounced in SLE patients, and that the inflammatory activity was central to the increased endothelial activation biomarkers and to endothelial dysfunction. Additionally, patients with RA have a much higher incidence of CV events than the incidence reported for the general Portuguese population, for which the inflammatory and endothelial activation markers contribute significantly.

In the SLE cohort, we evaluated how bone metabolism biomarkers, specifically OPG (osteoprotegerin) and RANKL (receptor activator of nuclear factor-ƙB ligand), are affected by the disease. We have found that these patients have an increased sRANKL/OPG ratio, due to

reduced OPG levels. Also, both OPG and sRANKL levels were associated with SLE specific antibodies, but not with corticosteroids therapy, suggesting that the increased osteoclastic stimuli are driven by SLE disease mechanisms.

Finally, we intended to comprehend the role of inflammation as common contributor to the interplay between bones and vessels. For that we have first used a mouse model of atherosclerosis ($ApoE^{-/-}$) and evaluated bones and vessels disturbances and verified their association with inflammatory markers. Our results suggest that inflammation is not the principal driver for atherosclerosis progression and bone disturbances in this animal model, but that altered lipid transport and high-fat diet, associated with mice aging, may play a more significative role.

Secondly, using bone and vessel samples from organ donors and atheroma plaques from endarterectomized patients with advanced atherosclerosis, we have evaluated the role of inflammation in the relationship between bone changes and vascular pathology. Our results suggest that the relationship between the changes observed in bones and vessels in the context of atherosclerotic disease and osteoporosis, may rely on the intrinsic connection between the tissues involved, independently of the progression of both diseases.

Overall, our results suggest that the connection between atherosclerosis and osteoporosis rely on an intrinsic connection between the tissues and that the contribution of the risk factors for the diseases progression, including inflammation, affect more pronouncedly patients with immune-mediated rheumatic diseases.

Keywords: Atherosclerosis, Osteoporosis, Inflammation, Immune-mediated Systemic Diseases, Cardiovascular Diseases

INTRODUCTION

Cardiovascular diseases (CV) and osteoporosis (OP) are among the most prevalent health problems, significantly contributing to increased morbidity and mortality of the population. According to the World Health Organization (WHO), CV diseases are the first cause of death worldwide [1], and atherosclerosis is the major cause of CV events [2]. OP is the most common human bone disease, characterized by decreased bone mineral density (BMD) and increased fracture risk due to low bone mass and structural deterioration of bone [3,4]. The incidence of these conditions increases with age and they frequently coexist in the same patient. Epidemiological studies have clearly demonstrated that in patients with CV diseases, the risk for osteoporotic fracture is 1.2 to 6.7 times higher than in general population [5]. This relationship is also observed in patients with low bone mass who have an increased risk for CV events, vascular calcification and atherosclerosis progression [6,7]. This association is particularly relevant in patients with chronic inflammatory, immune-mediated diseases like Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA) that have increased rates of subclinical atherosclerosis [8] with respectively, a 5-8 and 2-3 fold higher risk of CV events [9], and an increased prevalence of OP of about 5-25% in SLE [10,11] and 15-35% in RA [12].

Vessel inflammation and atherosclerosis

Atherosclerosis is the most common pathologic process that underlies CV diseases [2,13,14]. Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of lipids, inflammatory cells, fibrous elements, cellular waste products and calcium [5,15] in sub-endothelial regions, leading to narrowing of the arterial lumen (stenosis), which can evolve to atheroma plaque formation, plaque rupture and thrombosis. Atheroma plaques and progressive stenosis affect mainly large and medium-sized arteries [13,16,17]. As a consequence, the risk for CV events, like ischemia of the heart, brain or extremities increases [18], particularly in the presence of instability of the atherosclerotic plaque [19]. It begins with a disruption and activation of endothelial cells (ECs) with increased surface adhesion molecules (including ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1)) leading to augmented vascular permeability that allows the attachment and infiltration of lipids and inflammatory cells into the sub-endothelial space. The increased connection of low-density lipoprotein (LDL) to endothelium and smooth muscle cells (SMCs) raises the transcription of the LDL-receptor gene, which is activated by proinflammatory cytokines like tumor necrosis factor (TNF) and interleukin (IL-) 1β [20]. Oxidative modification of LDL is believed

to have an important role in atherogenesis. Even slightly changed LDL may induce the expression of adhesion molecules and cytokines by ECs. Increased formation of oxidized LDL in the vascular intima might lead to monocyte recruitment [21]. The initial sign of atherosclerosis is EC dysfunction/activation in response to oxidized lipids in the subendothelium, and expression of VCAM-1. This will start both the adhesion of leukocytes and the migration of activated platelets into the endothelium. Monocytes and T cells bind to ECs expressing VCAM-1 and migrate into the arterial tissue [22]. Consequently, there is an increase in the production of cytokines and growth factors, lipid accumulation, SMCs proliferation [15] and up-regulation of adhesion molecules, with subsequent infiltration and diapedesis of monocytes through the arterial intima, leading to the formation of pro-inflammatory foam cells and atherosclerotic plaque [16]. These inflammatory cells, specifically monocytes, will differentiate into macrophages, which will then accumulate lipids forming the foam cells [22] and produce and release pro-inflammatory cytokines (e.g. IL-1β and IL-6), creating an inflammatory environment and perpetuating this cycle [18]. In early lesions, vascular smooth muscle cells (VSMCs) proliferate/migrate and form a fibrous cap that encloses the growing lipid core, forming a more stabilized plaque. Recruitment, activation and differentiation of monocytes into macrophages and dendritic cells (DCs) are induced by the initial vascular activation, which is quickly followed by an adaptive immune response and recruitment of T cells [23]. Intima infiltration by activated T lymphocytes (CD4+, HLA-DR+ e IL-2R+), although in lower number than macrophages, has regulatory key functions on atherosclerotic plaque [24]. B lymphocytes and mast cells are scarce in the intima, but may be seen in the adventitia at sites of atherogenesis [25]. However, the gradual loss of VSMCs by apoptosis and increase activity of matrix-degrading enzymes in the cap, can result in plaque rupture, with subsequent platelet aggregation and thrombosis (Figure 1) [26]. So, the process is more complex than just a passive accumulation of cholesterol in the vessel, as previously thought [24,25].

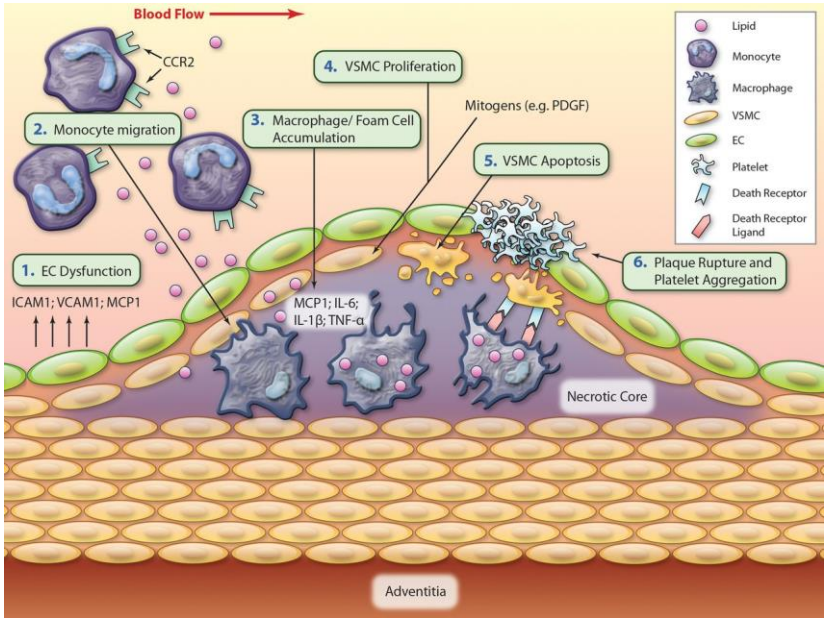

Figure 1 – Representation of atherogenesis and an unstable atherosclerotic plaque

Plaque formation involves a series of events: (1) EC dysfunction and activation; (2) attachment and infiltration of lipid and inflammatory cells into the subendothelial space; (3) Monocytes differentiate into macrophages, foam cells formation and release of proinflammatory cytokines; (4) VSMCs proliferation and fibrous cap formation, enclosing the growing lipid core; (5) VSMCs apoptosis and gradual degradation of the fibrous cap; (6) plaque rupture, with subsequent platelet attachment and thrombosis. (Adapted from Wang and Bennett, 2012) [26]. EC – Endothelial cell; VSMC – vascular smooth muscle cell; ICAM-1 - intracellular adhesion molecule 1; VCAM-1 - vascular cell adhesion molecule 1; MCP-1 - monocyte chemotactic protein 1; IL – Interleukin; TNF – Tumor necrosis factor.

The highest impact of atherosclerotic disease occurs in adults over 45 years old [15] and constitutes one of the top death causes in industrialized nations [27]. Atherosclerosis and its complications are the leading cause of death and productive loss of life in the Western world. This disease progresses silently over decades. There is even evidence of early vascular changes during the fetal period [21]. Atherosclerosis is a multifactorial disease dependent on environment, lifestyle, nutrition and other specific individual characteristics, more than just genetics and age.

Several risk factors for atherosclerosis have been identified. In addition to high plasma concentrations of LDL, there are other causes, such as free radicals caused by cigarette smoking, hypertension, *diabetes mellitus* and genetic alterations that lead to endothelial dysfunction, which is the initial step of the atherosclerotic process [17,28]. This causes the development of pores in inter-endothelial junctions. Then, the injured or dysfunctional endothelium develops a surface that is conducive to inflammatory cells adhesion, rolling and migration to subendothelial region [20]. Even though LDL appears to be a significant factor, some patients may develop CV diseases without changes in lipid profile [15].

Epidemiological studies unequivocally showed an increased vascular risk in individuals with high levels of proinflammatory cytokines [29,30] and acute phase reagents [31–34]. The elevation of inflammatory parameters confers a risk similar or superior to that presented by hypercholesterolemia.

In addition, there is evidence that several infectious agents aggravate CV risk, probably exerting their effect through inflammatory mechanisms [35,36]. In support of the relevance of systemic inflammation are also the results of clinical trials demonstrating a significant reduction of CV risk obtained with statins in individuals with elevated inflammatory parameters [37].

The relationship of atherosclerosis with conditions like type 2 diabetes and obesity is also partially explained by interactions with inflammatory and immune mechanisms occurring in these diseases [38]. The adipose tissue is responsible for the secretion of several hormone-like molecules called adipokines. These molecules regulate the lipids and carbohydrates metabolism and act in the inflammatory response, immune regulation and on angiogenesis, inducing low-grade inflammation and having pro-inflammatory and atherogenic effects (e.g. leptin, resistin and ghrelin). This regulation is complex and at least one adipokine, adiponectin, has been associated with a vascular protective effect, showing an anti-inflammatory and anti-atherosclerotic action [14,39] in the context of chronic inflammatory/immune-mediated diseases (SLE and RA, for example) [40]. This possible anti-atherogenic effect was recently corroborated by the negative association between serum adiponectin levels and intima-media thickness in a large study [41].

Leptin and adiponectin are the best-studied adipokines in their relationship to atherosclerosis. Although there is some divergent data [42], most studies show a pro-atherogenic effect of leptin, while adiponectin has been reported to have an athero-protective effect [43,44].

The key mechanism in atherosclerotic disease is inflammation [45], comprising a complex interaction with various cell types and cytokines that relate CV risk factors with immune-inflammatory activation of the vascular wall [23]. Inflammation is present and affects the progression of the disease during all phases [27], from fatty streaks and plaque formation to an acute episode caused by plaque erosion/rupture and subsequent thrombosis [16,20]. As an inflammatory disease, endothelial dysfunction and inflammatory lesions are mediated by a number of pro-inflammatory cytokines. There is evidence of an increase of IL-1β, IL-6 and TNF [13,46,47] that contribute to lesion progression [25] and are associated with an aggravation of CV risk, as demonstrated by epidemiological studies [48].

Innate and adaptive immunity operates in the development of atherosclerosis since immune cells and their mediators lead to chronic arterial inflammation. Macrophages, T lymphocytes

and mast cells are responsible for the latent inflammatory response in the vessel wall [22]. Pro-inflammatory cytokines present in atherosclerotic plaques are produced by monocytes and macrophages [49].

Increased serum levels of proinflammatory cytokines favor activation and dysfunction of ECs. TNF, IL-1 and INFγ promote the expression of adhesion molecules by ECs, the recruitment and activation of inflammatory cells and may be responsible for triggering the cascade of inflammation in the arterial wall [50]. IL-17, activates several downstream pathways, such as induction of adhesion molecules, like ICAM-1. However, IL-17 also downregulates the expression of endothelial VCAM-1. IL-17 may vary its effects on CV disease depending on the cell type that produces it and the environment where it acts, suggesting that IL-17 could have both pro- and anti-atherogenic roles [22,23]. Fibrinogen, often elevated in inflammatory diseases, by binding to vascular receptors such as ICAM-1, acts as a key link to increase adhesion and transendothelial migration of leukocytes [51].

The pro-inflammatory cytokine IL-1β, produced by monocytes and macrophages, is responsible for activation and up-regulation of ECs and leukocyte adhesion molecules, proliferation of VSMCs and secretion of other cytokines associated with the progression of atherosclerosis and, in some human studies, high expression of IL-1β was found in arteries with atherosclerotic lesions [16]. IL-1β is implicated in several diseases, such as diabetes and leukemia but also in diseases with a strong inflammatory component like inflammatory bowel disease, RA [52] and also, atherosclerosis [53]. In fact, according to the CANTOS study [54], IL-1β inhibition with canakinumab reduces hs-CRP and IL-6 levels, as well as the incidence of myocardial infarction, stroke and CV death, without any detectable impact on lipids plasma levels [55,56]. Il-1β often acts together with IL-6, another essential cytokine in the progression of atherosclerotic lesions and its rupture, triggering inflammation and expansion of inflammatory response in atherosclerosis and other chronic systemic inflammatory diseases. It is secreted into the lesions by macrophages, ECs, fibroblasts and adipose tissues. Its actions lead to endothelial dysfunction, VSMCs migration and transition of macrophages into foam cells [16]. The activation of the inflammatory process leads to interaction with other molecules that modulate various mechanisms in other tissues, e.g. adipose tissue and blood vessels, increasing the plasma levels of inflammatory mediators like TNF and C-reactive protein (CRP), among others [57,58]. TNF is also involved in the pathogenesis of systemic inflammatory diseases and there is evidence of involvement in atherogenesis and vascular inflammation. The main producers of this cytokine are macrophages, monocytes, ECs, VSMCs and fibroblasts. It binds to its two receptors, TNF receptor I (TNFR1) and TNF receptor II (TNFR2) and this process induces the activation of nuclear transcription factor (NF)-kB. Its role in atherosclerotic disease

is mediated by an increase in the expression of vascular endothelial adhesion molecules, recruitment of macrophages and leaking of microvasculature. Moreover, it regulates nitric oxide synthesis and reactive oxygen species formation that cause endothelial dysfunction. In fact, some studies reveal that TNF administration leads to an increased expression of ICAM-1, VCAM-1 and E-selectin and production of reactive oxygen species [16]. Cytokines such as TNF can be up-regulated by NF-kB, which is activated under oxidative stress, creating a vicious cycle and maintaining a chronic low-grade inflammatory activity in the vessel. [25,59,60].

Chronic inflammation makes an important contribution to the proliferation of SMCs and remodeling of the vascular wall. However, the mechanical stimuli to which the endothelium is directly exposed (e.g. bloodstream forces) is another vector of vascular remodeling. In the case of endothelial injury, mechanical stimuli are perceived directly by SMCs, inducing profound changes in their properties. Middle layer cells and adventitial fibroblasts also have the capacity to undergo rapid phenotypic transformations, proliferate and migrate. In response to aggressions, SMCs produce extra-cellular matrix, including collagen and elastin, thus contributing to neointima and fibrous plaque formation. The progression of lesions, potentiated by various injuries to the vessel wall, is characterized by focal necrosis and by a repairing fibroproliferative process that reduces the vascular lumen (atheroma plaque), alters blood flow and causes chronic tissues ischemia. If there is thrombosis or embolism of the plaque there is a sudden interruption of the blood flow that gives rise to an acute ischemic event. Thrombosis is triggered by plaque rupture and exposure of thrombogenic material leading to platelet aggregation and activation. The most vulnerable plaques predisposed to rupture and atherothrombosis also present an intense inflammatory infiltrate [61].

The endothelium is the main regulator of the vascular wall, responsible for maintaining the balance between vasoconstriction and vasodilation, between stimulation and inhibition of proliferation and migration of SMCs and between thrombosis and fibrinolysis. Endothelial function is compromised in individuals with established atherosclerotic disease [62], but alterations in endothelial function are also an early marker of vascular disease, and may precede structural changes in the arterial wall [63]. The presence of CV risk factors is a strong predictor of endothelial dysfunction [64] and subclinical inflammation correlates with endothelial changes [65]. These changes are potentially reversible with pharmacological therapies (statins, cholestyramine, angiotensin converting enzyme inhibitors or hypoglycemic agents) and non-pharmacological (diet or exercise) [66,67].

As the plaque develops, deposition of calcium will also occur. This can happen in two ways: some authors defend that the increase of inflammatory cytokines will provide a VSMCs reaction, which will migrate, proliferate and turn into osteoblasts-like cells and will cause

instability and calcification of the atherosclerotic plaque [17]. Others believe that this mechanism is more like an endochondral bone formation due to chondrocyte-like phenotype expressed by VSMCs. This endochondral bone formation consists in the differentiation of a cartilage model, which is posteriorly invaded by osteoblasts and replaced by mineralized bone [68].

Bone inflammation and osteoporosis

Bone is one of the most dynamic human tissues, as it remains metabolically active throughout life and has pleiotropic functions in the organism. It not only protects the internal organs, supports the body and assists in its movement, but also functions as a mineral (calcium and phosphorus) and fat reservoir and harbors bone marrow, where hematopoiesis occurs [69].

Bone is composed of three different cell types: osteoclasts (OCs), osteoblasts (OBs) and osteocytes. These cells are responsible for the daily bone renewal in a tightly balanced process [70–72]. On the other hand, the organization of bone as a tissue is dependent on complex interactions between three main players: type I collagen, hydroxyapatite crystals and cells [72].

From a structural perspective bone can be classified in hierarchical levels of complexity, all affecting its mechanical properties. It must be stiff, slightly flexible and light to make loading possible and facilitate movement [72–74].

Additionally, bone has a unique characteristic that distinguishes it from other tissues: it can respond according to the location and extent of the biomechanical loading and microdamage, remove the damaged tissue and replace it with new bone [72,75,76]. Disequilibrium of this system is observed in bone metabolic diseases, such as primary OP, or as consequence of systemic inflammatory diseases such as RA [72,77]. This will induce bone fragility and eventually low energy fractures [78].

The bone mechanical properties describe the relationship between applied loads or forces (designated as stress) and bone deformation. Deformation is referred to as strain and is defined as a relative change in size or shape. Normal strains represent elongation or shortening while shear strains represent distortion. Therefore, a load-deformation curve or a stress-strain curve are often used to define the biomechanical properties of bone [79].

The load-deformation curve reflects the structural behavior of bone and describes the amount of load needed to produce a unit of deformation. The analogous stress-strain curve reflects the material behavior of bone tissue and this curve is generated through a mechanically standardized (size and shape) test. Load and deformation have a linear relationship until the

yield point is reached; at this time the slope of the curve is reduced. Before the yield point the material obeys to the Hooke's law, which states that there is a proportional relationship between stress and strain. This linear relationship is translated by the Young's modulus, or bone stiffness, that measures the resistance of the bone to deformation [80,81]. The yield point is the cross point of transition from an elastic behavior to a plastic one and corresponds to the occurrence of the first microfractures in bone tissue. The slope of the elastic region defines bone stiffness, while the strain energy, or toughness, is the energy required to initiate failure of the structure and is computed as the area under the curve [79,81].

Bone tissue is composed of two main patterns, cortical and trabecular bone. Cortical bone represents 80% of skeletal bone and is dense and compact with low turnover ratio; it constitutes the outer part of all bones, providing mechanical strength and protection [82]. The trabecular bone, also known as cancellous bone, only composes 20% of the whole skeleton and is a spongy-like tissue present in the interior of the bone, highly vascularized and is formed by a porous network called trabeculae [83]. It is light, quickly adaptable to external biological stimulus, flexible and capable of absorbing energy [82]. Even though representing only 20% of bone, its remodeling rate is much higher than cortical bone.

The bone matrix comprises an organic and an inorganic phase. The organic phase is composed mainly by type I collagen, glycoproteins, proteoglycans and bone cells. On the other hand, the inorganic part is formed by carbonated hydroxyapatite crystals ($Ca_{10}(PO_4)_6(OH)_2$) that are distributed among the collagen fibers [80,84–86]. The composition and organization of the bone matrix gives this tissue unique mechanical properties such as stiffness, ductility, tensile strength and exceptional lightness [86,87]. In vivo, bone is subjected to several different loading modes producing tension, compression, shear, bending, torsion and combined loading. In bending, a combination of tensile and compressive loads is applied in such a way that causes the structure to bend. Bending tests can be produced by three (three-point bending) or four (four-point bending) forces and are mostly applied to long bones [88,89].

The organic component of bone is comprised by type I collagen (90%) and noncollagenous proteins. Type I collagen is synthesized by OBs. It self-assembles in fibrils and fibers that are deposited in parallel or concentric layers [90–92]. There are several non-collagenous proteins on bone, such as phosphoproteins, proteoglycans, glycosylated proteins and γ-carboxylated proteins. These include osteocalcin (OCL), matrix gla protein (MGP), osteopontin (OPN), bone sialoprotein (BSP), alkaline phosphatase (ALP) and osteonectin [79,93]. These proteins are all involved in the ordered deposition of hydroxyapatite by regulating the amount and size of the mineral crystals [91,93–96].

The type I collagen is the principal protein of bone, a heterotrimer comprising α1 and α2 protein chains encoded by the *COL1A1* and *COL1A2* genes. *COL1A1 Sp1* polymorphism is associated with osteoporotic fractures and the density of bones [4].

In clinical practice, products of the collagen processing or breakdown, either for the formation or degradation, act as markers of collagen turnover and are used to assess bone remodeling [97]. Type I collagen propeptides, amino-terminal (P1NP) and carboxy-terminal (P1CP) result from post-translational cleavage of the pro-collagen molecule before its organization in fibrils [98]. The propeptides P1NP and P1CP are two small domains present in the procollagen molecule, which after secretion of the molecule into the extracellular space are enzymatically cleaved. Since both propeptides are stochiometrically produced from each new collagen molecule, P1NP and P1CP are considered quantitative measures of collagen formation [99]. Moreover, P1NP serum levels correlate with bone formation indices assessed by histomorphometry [100–102]. The strength of the collagen fibers depends on the formation of covalent cross-links between the telopeptides and the adjacent helical domains of collagen molecules. The cross-linked telopeptides of type I collagen, amino (NTX) and carboxy-terminal (CTX), are products of the collagen cleavage. Both NTX and CTX are products of cathepsin K (CTSK) action and represent collagen breakdown [101–104].

Osteocalcin is exclusively expressed in bone and dentin. It is a calcium-binding protein characterized by three gamma-carboxyglutamic acid residues. Due to its interaction with hydroxyapatite it is believed that OCL regulates the growth and maturation of bone crystals [105]. In fact, OCL measurements in serum have proven to be a valuable marker for bone turnover in metabolic bone diseases [97].

The endpoint for matrix mineralization is the deposition of crystals between collagen fibers, a process that comprises two phases, an initial formation of apatite crystals within matrix vesicles and a subsequent propagation phase within the matrix [106].

Both collagen and non-collagenous proteins regulate the expansion of crystals, while cells can affect their maturation status [106].

There are three main cell types present in bone: (i) OCs, giant multinucleated cells derived from macrophage-monocyte lineage that resorb bone by dissolving the mineral phase and by enzymatically degrading extracellular matrix proteins; (ii) OBs, cells of mesenchymal origin responsible for bone formation that are able to produce the organic bone matrix and aid in its mineralization; (iii) and osteocytes, that are mature OBs that become entrapped in the bone matrix and act as mechanosensors, a crucial function in the regulation of bone remodeling [85,107,108].

Bone resorption and formation not only should be quantitatively balanced but must also be coupled in time and space. This tight coupling is essential for the repair of microscopic damages that result from constant impact [109]. Bone remodeling (Figure 2) is a dynamic and continuous process that occurs throughout life, in which old bone is replaced by new one through the action of OBs, OCs and osteocytes [110,111]. These cells have a central role in the maintenance of the skeleton mechanical integrity and repair of fractures [111].

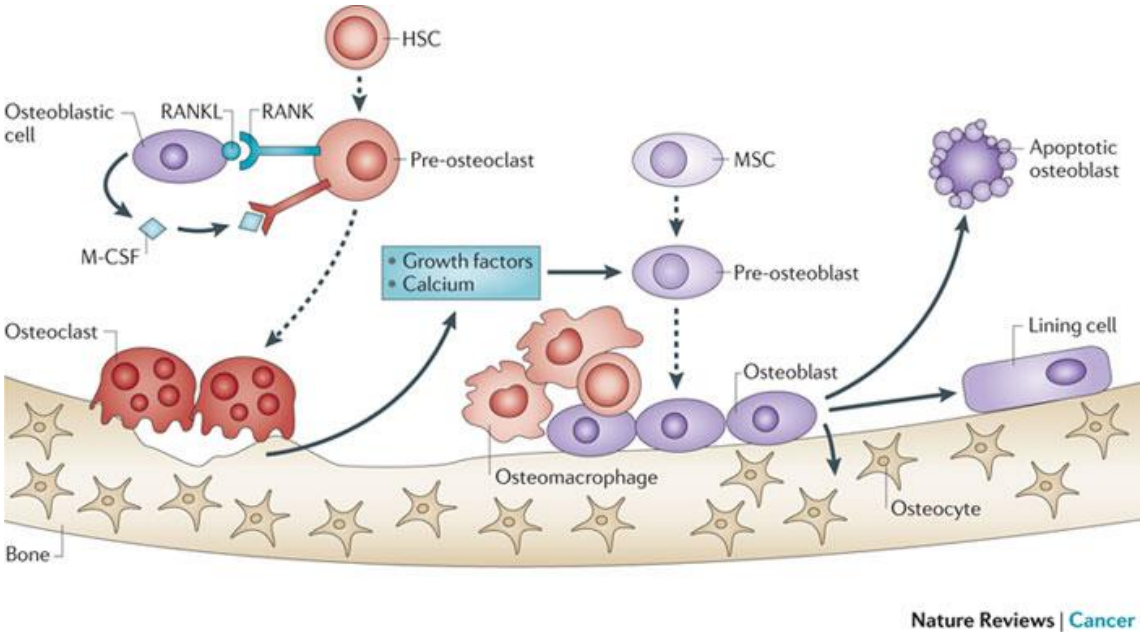

Figure 2 – Representation of bone remodeling

Osteoclasts (OCs) are derived from hematopoietic stem cells (HSCs) and become activated by RANK-RANKL binding and M-CSF stimuli, acquiring the ability to resorb bone and release growth factors and calcium, allowing mesenchymal stem cells (MSCs) differentiation into osteoblasts (OBs). OBs will replace the cavities with new bone, with the production of type I collagen and its subsequent mineralization to form the calcified matrix of bone. Osteocytes, terminally differentiated OBs embedded in bone, sense mechanical strain and signal to OCs and OBs, thus participate in the remodeling process. (Adapted from Weilbaecher K. et al., 2011) [112]. M-CSF - macrophage colony stimulating factor; RANK - receptor activator of NF-κB; RANKL - RANK ligand.

OCs, derived from hematopoietic lineage [109], are the unique bone resorbing cells, formed by cytoplasmic, but not nuclear, fusion of mononuclear precursors of the monocyte/macrophage lineage of the bone marrow (hematopoietic stem cells - HSCs), peripheral circulation and tissue macrophage populations [113–116]. Osteoclastogenesis is a complex process that includes many stages, such as commitment, differentiation, multinucleation and activation of immature OCs [117,118]. OCs formation, differentiation, function and survival are regulated by a network of

signaling pathways and depend on two major factors: macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (RANK) ligand (RANKL) [115,119–121].

M-CSF is an hematopoietic growth factor expressed by OB lineage cells involved in the proliferation, differentiation and survival of monocytes, macrophages and bone marrow progenitor cell [122,123]. M-CSF interacts with CSF1R expressed on OC precursors, which is essential for their proliferation and survival and stimulates surface expression of RANK [124,125].

RANKL is a member of the TNF superfamily. It is expressed by bone marrow stromal cells, OBs, osteocytes, chondrocytes and immune system cells and interacts with its receptor RANK, expressed at the cell surface of OC precursors to induce OC differentiation [119,126–130]. RANK is a type I transmembrane receptor protein, member of the TNF receptor superfamily, expressed primarily on the cells of the monocyte/macrophage lineage including OC precursors and DCs but also on B and T cells and neutrophils [119,131–133].

RANKL exists both as soluble and membrane-bound forms [93]. The soluble form (sRANKL) corresponds to the c-terminal part of membranous RANKL that may be produced either directly by the stromal cells or OBs through alternative splicing followed by secretion to the extra-cellular medium, or by proteolytic cleavage of membranous RANKL by TNF- α converting enzyme (TACE) [110]. Membrane-bound RANKL is expressed at the cell surface of OBs, bone marrow stromal cells, fibroblasts, mammary epithelial cells and activated immune system cells [124]. Deletion of the RANKL gene in mice resulted in severe osteopetrosis and a complete lack of OCs, as a result of an inability of OBs to support osteoclastogenesis [134].

RANKL also binds to another TNF receptor family member, osteoprotegerin (OPG), which is produced by OBs, bone marrow stromal cells, immune system cells [124,135,136] and expressed in normal VSMCs and downregulated in calcified VSMCs [137]. OPG was first identified in 1997 as being a protein that exhibits a protective effect on bone [138], although it is expressed ubiquitously and abundantly in many tissues and cell types [139]. When bound to RANKL, OPG prevents its binding to RANK and thus inhibits the biological activity of RANKL, being the major negative regulator of bone resorption [119,140–143]. It also exhibits a protective effect against calcification in cells by reducing alkaline phosphatase [137]. $OPG^{-/-}$ mouse exhibit a decrease in total bone density with severe trabecular and cortical bone porosity, thinning of the skull, and a high incidence of fractures [144], contrary to OPG overexpression that causes severe osteopetrosis in mice [138].

These studies demonstrated the importance of the RANKL/OPG ratio in OC differentiation and on its bone-resorptive function [125]. The amount of RANKL and OPG expressed by OBs depend on their stage of differentiation: pre-OB cells express high levels of RANKL and relatively low levels of OPG, thus stimulating OC differentiation and function. On the other hand, more

mature OBs express higher levels of OPG, in comparison to RANKL levels, inhibiting OC differentiation and function [145]. Hence, the RANKL/OPG ratio in bone microenvironment is the main molecular mechanism that determines osteoclastogenesis.

Together, M-CSF and RANKL are required to induce expression of OC lineage-specific genes, including those encoding TRAP, CTSK, calcitonin receptor (CALCR) and αvβ3 integrin, leading to the development of mature OCs [113].

OC precursors differentiate into $TRAP^+$ mononuclear cells after RANKL stimulation. These cells fuse with each other and differentiate into multinucleated OCs [146]. Cell fusion is one of the most distinctive characteristic properties of OCs. The OC also contains a unique and complex ruffled membrane, which is the resorptive organelle. Although the origin of this specialized membrane was enigmatic for some time, it is now considered to be the most specific marker of the OC, since it appears only when the cell is resorbing bone [147–149]. Mature OCs express high levels of tartrate-resistant acid phosphatase (TRAP), which has been widely used as a cytochemical marker of OCs and their precursors [150] and several other genes that regulate their resorptive ability, including those encoding the chloride/H^+ antiporter channel CLCN7 and CTSK [115,120]. TRAP is a soluble acid resistant phosphatase secreted by the OC that plays a role in bone resorption [151].

Bone resorption is a complex and specific process that occurs in three stages: (i) OC attachment to bone matrix and cell polarization, (ii) resorption, and (iii) cessation of resorption. The first study about the basic mechanism of bone degradation was performed in 1985 and established that the resorptive space is a highly acidified microenvironment [183]. Bone collagen degradation occurs through the action of a lysosomal enzyme, CTSK, which also polarizes to the ruffled membrane upon OC attachment to bone [152]. After removal of the inorganic bone phase and exposure of the matrix proteins, procathepsin K is activated. CTSK is secreted by OCs to degrade several bone matrix proteins, among which are included type I collagen and OPN. CTSK degrades type I collagen in its non-collagenous termini (N- and C-telopeptide regions) and releases crosslinked N- and C-telopeptides (NTX-I and CTX-I), which can be detected in urine and serum by immunoassays and are currently used to assess bone turnover [153,154].

OBs are mononuclear, non-terminally differentiated, specialized cells that derive from the mesenchymal stem cell (MSC) lineage [109,155] and have the ability to secrete bone matrix where hydroxyapatite crystals deposit [145,156,157]. OBs, chondrocytes, adipocytes, myoblasts, tendon cells and fibroblasts are all derived from MSC. The lineages are determined by specific transcription factors [157]. The differentiation of a MSC into a functional OB requires the presence of three transcription factors, core-binding factor subunit alpha-1 (Cbfa1, also known

as runt-related transcription factor 2 - Runx2), osterix (Osx) and Wnt/β-catenin [158]. More than a decade ago, Cbfa1 was established as essential for bone formation since mice deficient in this transcription factor showed no ossification [159]. Cbfa1 is a member of the runt family of transcription factors that is expressed by MSCs at the onset of skeletal development and is present in OBs throughout their differentiation [160]. Osx is another essential transcription factor for OB differentiation. Osx transcription is positively controlled by Cbfa1 [161]. Osx acts by forming a complex with NFATc1 (nuclear factor of activated T cell c1) resulting both in the activation of the type I collagen promoter [162] and in the induction of Wnt signaling pathway and bone formation [163].

The progressive development of the OB phenotype from a proliferating immature cell to a mature OB that is able to synthesize bone proteins is characterized by a sequential expression of specific genes that identifies three periods of OB phenotype development: proliferation, maturation and extracellular matrix synthesis, and matrix mineralization [164]. OB differentiation and function, and therefore bone formation, is under the control of bone morphogenic proteins (BMPs) and Wnt signaling pathways [107,158]. The Wnt signaling pathway regulates several aspects of OB activities, including commitment of MSCs, OB progenitor amplification and cell death. The first evidence that Wnt/β-catenin signaling plays an important role in bone formation came from studies in humans where mutations that inactivate the Wnt co-receptor low density lipoprotein receptor-related protein (LRP)5 were shown to cause OP [165].

In the absence of Wnt proteins, glycogen synthase kinase-3β (GSK-3β) phosphorylates β-catenin, which is degraded, and the OB signaling cascade is blocked, so the MSCs become chondrocytes or adipocytes [166,167]. The Wnt/β-catenin pathway is frequently referred to as the canonical pathway and it promotes cell fate determination, proliferation and survival through the increase of β-catenin levels and alteration of gene expression by the transcription factor lymphoid enhancer factor/T cell factor (LEF/TCF) [166,168,169]. Activation of this signaling pathway occurs with binding of Wnt to the receptor Frizzled (FZ) and the co-receptors LRP5/6 [170–172]. Some molecules specifically block the Wnt pathway such as secreted Frizzled related proteins (sFRPs), Wnt inhibitory factor (WIF), the dickkopf-related protein 1 (DKK1) and sclerostin (SOST) [172–175]. DKK1 is a soluble inhibitor of Wnt pathway produced by osteocytes and OBs [169]. It interacts with LRP5/6 and transmembrane proteins kremen1 and 2, leading to the internalization of this complex and degradation by the proteasome [176]. Its overexpression leads to inhibition of OB proliferation and impaired mineralization [173] and its blockade leads to increase in bone mass [177–179]. Another inhibitor of the Wnt pathway is SOST, a secreted protein mainly expressed by osteocytes [180]. This protein is secreted in response to mechanical stimuli, arresting bone formation, as shown in both animal model and human disease [181–185]. Like DKK1,

SOST inhibits Wnt/β-catenin pathway by binding to LRP5/6 co-receptor; however, it binds to a different region of LRP5/6 and it does not mediate receptor internalization [169,186]. SOST knockout mice have increased BMD, bone volume, bone formation and bone strength [182] while overexpression of SOST leads to osteopenia [180].

During the post-proliferative phase, which is characterized by high levels of ALP, the extracellular matrix progresses to the mineralization phase, in which OBs synthesize several proteins associated with the mineralized matrix [187] including BSP [188], OPN and OCL [189]. Collagen type I, ALP and OCL are specific phenotypic markers of OBs expressed during its differentiation [119]. OCL is expressed only in the post-proliferative phase, showing highest levels during mineralization [119,190].

Besides synthesis and deposition of extracellular matrix proteins OBs are also responsible for the synthesis and secretion of molecules responsible for initiate and control OCs differentiation [119]. OBs have the ability to regulate bone resorption through the expression of RANKL, which binds to its receptor, RANK, on the surface of pre-OC cells, inducing their differentiation. On the contrary, the soluble decoy receptor OPG, also produced by the OB, is able to block RANK/RANKL interaction by binding to RANKL and thus prevent OC differentiation and activation [191].

At the end of the bone formation phase OBs have one of three possible fates: they can be embedded in the matrix and differentiate into osteocytes, they can become bone lining cells or they can undergo apoptosis [158,192–195]. When OBs become entrapped in the bone matrix a dramatic shift in both shape and function occurs and the cell becomes an osteocyte. Osteocytes are non-proliferative, terminally differentiated cells and constitute the main cellular component of mammalian bone, representing more than 95% of bone cells. They reside both in mineralized matrix and in newly formed osteoid, locked inside small lacunae. Osteocytes have dendritic morphology [126,196,197]. Once embedded into the bone matrix, the osteocyte ceases its matrix synthetic activity and initiates the function as strain and stress sensor [198]. Another function of osteocytes within the bone cell network is the ability to deposit and resorb bone around the lacuna in which they are housed, thus changing the shape of the lacuna. This process is called osteocytic osteolysis and is limited to specific situations [199]. Osteocyte differentiation is accompanied by progressive reduction of several bone markers such as ALP, BSP, OCL, type I collagen and Cbfa1 [198,200]. Although osteocytes by themselves do not resorb or form bone, except in the lacunar area, they signal OBs and OCs to perform their functions [201]. Osteocytes have been shown to produce RANKL and M-CSF and to support the generation of functional resorbing OCs from their progenitors [202,203]. These cells also express OPG, influenced by β-catenin signaling [204,205]. Mechanical pressures and loads are sensed by

osteocytes and these cells are able to respond by modulating the expression and secretion of many molecules, including Insulin-like growth factor (IGF)-I, IGF-II, OCL [206] and type I collagen [207]. Osteocytes also produce two important inhibitors of OB proliferation and differentiation: SOST [180] and DKK1 [173,208].

Bone is continuously remodeled to ensure mineral homeostasis and to maintain the integrity and strength of the structure. Bone remodeling occurs at the BMU (basic multicellular unit) level and involves several sequential steps, beginning with OC formation, OC-mediated bone resorption, a reversal period where the matrix will be prepared for the next phase and a long period of bone matrix formation mediated by OBs, followed by mineralization of the matrix [192,209,210].

The correct balance between bone deposition and resorption is crucial for the proper maintenance of bone mass and loss of this coupling mechanism is the starting point for several skeletal pathologies [77,211], such as OP, characterized by low BMD and increased resorption, or osteopetrosis characterized by excess bone formation that leads to high BMD [212].

OP is a skeletal condition characterized by an imbalance in bone turnover with loss of bone mass and deterioration of bone microarchitecture resulting in decreased BMD and increased bone fragility. OP is a major risk for fragility fractures, causing substantial morbidity mainly amongst the elderly [4,108,213–215].

About 40% of postmenopausal Caucasian women are affected by OP and in the context of an aging population, this number is expected to increase significantly over the years. The patient with OP has an increased lifetime fracture risk, which most commonly occur in the spine, hip or wrist [126].

OP and consequent fractures are a serious public health problem and a significant cause of morbidity and mortality with a tendency to become worse due to the increase of life expectancy. Accordingly, 30-50% of women and 15-30% of men will suffer a fracture related to OP in their lifetime and, by 2050, the worldwide incidence of hip fractures will increase by 310% in men and 240% in women. The risk of death can be amplified by 20% to 24% during the first year after a hip fracture and the majority of those who survive can become bedridden. This disease is more common in the European countries but its prevalence is increasing in developing countries in Asia and Latin America [119,216]. Low BMD is a major risk factor for fractures. BMD determination is possible by performing a dual x-ray absorptiometry (DXA). The result is evaluated in T-score values, which is the number of standard deviations observed in the comparison between individual measured BMD and the mean BMD of a young population (bone mass peak). According to the WHO, OP is diagnosed if T-score < -2.5, ostepenia if T-score ≥ -2.5 and ≤ -1.0, and normal if T-score > -1.0 [217].

Primary OP is the most common type of OP, mainly defined as an age-related condition, characterized by bone mass loss increasing with age, leading to weakening of bones and fractures [218,219]. It is also associated with menopause, mainly due to estrogen deficiency that causes an impairment in bone turnover cycle, causing this disease to be more frequent in women that in men [219,220].

Secondary OP, on the other hand, has a direct identifiable cause. It is essentially associated with endocrine, metabolic or inflammatory diseases such as RA [221], SLE [222] and ankylosing spondylitis [223], or medications that cause bone metabolism imbalance [224]. Its complexity is due to its heterogeneous character, attributed to various endocrine, metabolic, and mechanical factors along with the usual risk factors such as age, menopause and certain lifestyle behaviors [108,215].

A relevant component of the fracture risk is hereditarily related and previous studies have shown that the value of BMD is 50% to 85% genetically determined [4].

Age-related bone loss affects both women and men. Significant trabecular loss begins in the early thirties in both genders but after menopause women experience acceleration in bone loss due to the rapid decline in estrogen after menopause [108]. In men, age-related changes in the levels of sex steroids, including both androgen and estrogen, also contribute to the pathogenesis of OP [225]. The variations in serum markers of bone turnover have been studied in a group of postmenopausal women showing that menopause induces a 79-97% increase in bone resorption marker levels and 37-52% increase in bone formation [226]. In early menopause, the acute phase of estrogen deficiency, the up-regulation of RANKL on bone-marrow stromal cells is associated with increased bone resorption [227,228]. In addition, estrogens also stimulate the expression of OPG in mouse OBs and stromal cells [229] and suppress the expression of TNF, IL-1 and IL-6 in monocytes, OBs and stromal cells [230]. Therefore, estrogens play a protective role in bone loss by modulation of the RANKL/OPG ratio and inhibition of pro-inflammatory cytokines secretion.

Rising molecular evidence suggests that inflammatory conditions with release of inflammatory cytokines like IL-1, IL-6, IL-17 and TNF can modulate bone by promoting (in the case of IL-1, IL-6, IL-17 and TNF) or inhibiting (IL-12, IL-33 and also the IL-6 which has this double function) osteoclastogenesis [231] as well as leading to alternative pathways within bone remodeling [60,231,232]. The term osteoimmunology was used for the first time in 2000 [233] to describe the interaction of cells from the immune and skeletal systems. This connection between bone and immune systems is not surprising, since precursors of immune cells reside in bone marrow, thus in the same environment as differentiated bone cells. Moreover, OCs are of hematopoietic origin. In addition, a new concept is emerging in which bone exerts a quality

control on immune responses by controlling the availability of HSCs [234]. Immune and skeletal systems have several regulatory factors in common, such as cytokines, transcription factors and receptors. Consequently, these two systems interact with each other, both in physiological and pathological conditions. Furthermore, as we age there is accumulation in the bone marrow of memory T cells, which express RANKL on their surface. It is believed that these cells might influence bone turnover and be responsible for some of the changes that occur in the skeleton with aging [235].

Atherosclerosis and Osteoporosis interplay

The association between vascular and skeletal system has gained prominence over the years, especially the paradox between vascular calcification and low bone density, supported by several studies in mice and humans.

Vascular calcification is a well-accepted marker of increased CV risk [236] and has been pointed as a possible predictor of CV events [5,237]. It is shown to be an active, cell-regulated process characterized by an ectopic mineral deposition in blood vessels, predominantly aortas. This mineralization can occur either in the tunica intima, associated with atherosclerosis (related with lipid accumulation and inflammation with focal plaque calcification) or in the tunica media of the vessel, a more generalized stiffening, known as arteriosclerosis or Monckeberg's medial calcified sclerosis (associated mostly with ageing, OP and other conditions like hypertension or *diabetes mellitus)* [68,238]. It was thought to be a passive, inevitable, natural process of ageing, but this deposition of minerals in the vessels was shown to be more complex and occurring in an organized manner. It is not a mere calcification, but an ossification-like process with deposition of mineral hydroxyapatite produced by calcifying vascular cells that have the ability to recapitulate the osteoblastic differentiation, leading to a bone-like structure in the vascular system [239]. This phenomenon is paralleled by the expression of several proteins implicated in normal bone development. Among these proteins believed to mediate arterial wall calcification stand OPG, RANKL, OPN, type I collagen and also pro-inflammatory cytokines [240], implicated in modulating adaptive immunity and considered as potential regulators of atherosclerotic lesions development and calcification. Despite RANK/RANKL/OPG system and the Wnt pathway being characteristic of bone metabolism, they have been identified in the development of atherosclerosis and could be a contributing pathway in regulation of vascular calcification [136]. Furthermore, both RANKL and OPG are

known to be expressed not only by bone-related cells but also by many components of the CV system, such as ECs, platelets, and VSMCs [236,241,242].

Previous studies have shown that increased OPG serum levels and its expression are associated with carotid atherosclerosis, myocardial infarction and with low BMD in postmenopausal women [243,244]. OPG serum levels are also related to atherosclerotic plaque growth, vascular calcification, increased risk of CV disease, peripheral arterial disease and CV mortality [245–248]. Animal model studies have demonstrated that $OPG^{-/-}$ mice, on top of being osteoporotic, have also shown an unexpected increase in vascular calcification [240], raising the hypothesis that OPG is a key regulator, not only of OP but also of vascular calcification [5,249]. In humans this association was also demonstrated in several epidemiological studies [250,251]. Clinical data have shown increased levels of OPG in association with endothelial dysfunction, vascular calcification, CV events and increased mortality [248]. RANK and RANKL expression by peripheral blood leukocytes is increased in patients with unstable angina, as well as monocytes / macrophages in thrombus removed from acute myocardial infarction (AMI) patients and on atheroma plaques in animal models, pointing to a likely role of RANK/RANKL on plaque instability [252]. In accordance, in RA patients, OPG levels correlate with coronary calcium score [253]. Although there is a strong association between these proteins and atherosclerotic lesions, the mechanism by which the calcification process is regulated is still unknown [248,254,255].

There are evidences linking atherosclerosis and CV mortality with low bone mass [9,256]. It is probable that OP and CV disease share common risk factors [5,256] as well as molecular and pathophysiological mechanisms [19,68,251,257]. Thus, there are possibly common underlying mechanisms shared by both conditions that are not yet fully understood.

Vessels, bones and systemic immune mediated diseases

Immune mediated rheumatic diseases, which include conditions such as RA and SLE, are inflammatory disorders that can involve multiple organs [258,259] and have as major comorbidities premature CV disease and OP, due both to underlying disease and chronic exposure to glucocorticoids [258–260].

Whereas the CV manifestations of immune-mediated disease can be mild and clinically silent, they can also increase morbidity and mortality substantially. Patients with systemic immune-mediated conditions often develop atherosclerosis, contributing to a higher mortality than in the general population, which is partially explained by an increase in the macrovasculature

endothelial damage, related to the persistent inflammatory response and associated with the presence of autoantibodies, immune complexes, and monocytes activation, although the precise etiology of endothelial damage initiation in an inflammatory context is still an enigma. Different components that are characteristic to the immunopathology of immune-mediated rheumatic diseases could be involved in the EC activation, increased permeability, functional alteration, and vascular injury [258,261].

Rheumatic conditions are associated with both local (periarticular) and generalized OP. Local bone loss is mainly due to the production of cytokines in synovial tissue. The systemic inflammatory response may affect the skeleton. In addition, therapy with cytotoxic drugs and glucocorticoids may further impair bone cell function [260], either through a direct effect on bone cells (upregulation of RANKL expression in OBs and downregulation of OPG in OCs, increasing osteoclastogenesis) or indirectly through its effects on muscle and calcium homeostasis [262].

Rheumatoid arthritis bones and vessels

RA is a chronic, systemic, immune mediated inflammatory disease that mainly affects the synovium of multiple joints, leading to the progressive destruction of cartilage and bone. This disease is characterized by pain, swelling and destruction of the synovial joints causing functional impairment and a significant increase of both morbidity and mortality [263,264]. Epidemiological studies have shown that RA affects about 1% of the world population and 75% of RA patients are women [265–269]. In Portugal, it has been reported that the prevalence of RA is 0.7% (95% CI 0.5%-0.9%) and it is also more frequent in women than in men [270,271]. Its incidence is highest among those aged between 30 and 50 years and, due to its chronic nature, the prevalence increases with age, as well as co-morbidities [266,268].

The predominant symptoms of RA are symmetrical pain, stiffness and swelling of peripheral joints. The clinical course of the disease is variable, ranging from mild to severe and highly destructive arthritis. The analysis of the clinical course and of laboratory and radiologic parameters have defined prognostic factors related to progressive joint destruction, such as the presence of autoantibodies like rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) [272–275]. In some patients these markers emerge years before the onset of the disease and probably contribute to its development and chronicity [276–279]. For classification purposes, mainly related to research, patients are classified as having RA based on the 2010

American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) RA classification criteria [280].

Evaluation of disease activity must be performed periodically. The disease activity score 28 joints (DAS28) is a frequent outcome measure used in clinical trials and is also used in therapeutic decisions. DAS28 is based on the count of tender and swollen joints (twenty-eight tender and swollen joints scores including: shoulders, elbows, wrists, metacarpophalangeal joints, proximal interphalangeal joints and knees), inflammatory activity (erythrocyte sedimentation rate, ESR or CRP) and the subjective evaluation of the global impact of the disease as measured by a visual analogue scale by the patient [281,282].

As a result of inflammation and structural damage there is a progressive decrease in functional capacity. The functional capacity measured by the Health Assessment Questionnaire Disability Index (HAQ-DI) is predictive of absenteeism, economic losses, early retirement and increased mortality [283]. Life expectancy is decreased in RA [284,285]. The causes of death of these patients are similar to those observed in the general population, but deaths occur 5-15 years earlier than would be expected in a population of the same sex and age but without RA [284].

Two of the most relevant RA comorbidities are OP and CV diseases. Indeed, RA patients have an increased risk of vertebral fractures, which is independent of BMD and corticosteroid use [286,287]. In addition, a high hip fracture risk was also noted in RA patients not exposed to corticosteroids [287]. Thus, RA itself seems to predispose to fractures as it is translated in the fracture risk assessment tool (FRAX®) [288]. On the other hand, CV diseases are the leading cause of death of RA patients. CV diseases account for about 40-50% of deaths, a value that has remained stable over the past few decades. Contrary to what is observed in the general population, deaths from CV disease remained unchanged in RA patients [264] and this reality contributes to an increasing mortality gap between this disease and the rest of the population [289]. In addition, CV events occur about a decade earlier in RA [290], suggesting that this disease, as in *diabetes mellitus*, is an independent risk factor for premature atherosclerosis [291,292]. RF-positive but not RF-negative RA patients are at increased risk of CV events following exposure to glucocorticoids [293].

RA is a complex multifactorial disease with unknown etiopathogenesis. In genetically susceptible individuals, specific environmental factors such as smoking habits, trauma or infection can activate potentially pathogenic immune pathways, contribute to disease development and its perpetuation, leading to chronic inflammation, joint destruction and systemic manifestations [265]. Among environmental factors, the potential contribution of smoking is highlighted. Tobacco predisposes to the transformation of arginine into citrulline and this post-translational modification can make several peptides more immunogenic and

contribute to the production of ACPA [294]. Other environmental stimuli, such as periodontal infection by *Porphyromonas gingivalis*, may interact with genes and trigger an autoimmune process [295]. In addition to ACPA and RF IgM (Immunoglobulin M), the production of other autoantibodies is documented, supporting the importance of B cells in the pathogenesis of RA.

The signs and symptoms of RA result from synovitis, the inflammation of the synovial membrane within joints. A complex network of cells and cytokines are involved in the pathogenesis of RA, particularly in the recruitment, activation and effector functions of immune cells. The synovial layers become vascularized and infiltrated with macrophages and fibroblasts in addition to an influx of B and T lymphocytes, plasma cells, mast cells, DCs and neutrophils [268,296].

In rheumatoid joints, it is well known that the imbalance between pro- and anti-inflammatory cytokine activities contributes to autoimmunity, chronic inflammation and therefore joint damage [297–299]. TNF [300,301], IL-1β [302,303] and IL-6 [304,305] are among the most important mediators of RA pathogenesis. T lymphocytes and other immune system cells produce pro-inflammatory cytokines such as TNF, IL-1β, IL-6 and IL-17A that enhance osteoclastogenesis and activate OCs [265,306]. Cytokines, such as TNF and IL-17, released from activated T lymphocytes, can target bone lining cells and fibroblasts and increase RANKL production, promoting OC activity [298,307]. Synovial T lymphocytes from RA patients produce low amounts of IFNγ but secrete TNF that induces RANKL expression in synovial fibroblasts. Moreover, RANKL is also expressed by activated T lymphocytes, of which Th17 are a major source [306,308,309]. IL-6 induces OC differentiation by promoting RANKL expression [310]. Blockade of IL-6 receptor reduced OC differentiation and bone resorption in monocyte cultures stimulated with RANKL [311]. IL-1β is a stimulator of TNF receptor-associated factor (TRAF)6 expression in the OC, thereby potentiating RANKL-RANK signaling cascade [312]. IL-1β can also indirectly facilitate osteoclastogenesis by acting on the OB inducing RANKL expression [313].

There are some similarities between RA and CV disease regarding the chronic inflammatory processes. The concentrations of CRP, IL-6 and TNF are elevated, and the cellular recruitment and activation patterns are alike. The pro-inflammatory molecules exert deleterious effects on the vascular endothelium, which subsequently reduces the synthesis of nitric oxide and promotes endothelial dysfunction [314,315], which can occur in both large and small vessels of the microvasculature, independently of disease activity or duration [316,317]. Also, these pro-inflammatory molecules induce alterations of further potential CV risk factors such as dyslipidemia, insulin resistance and oxidative stress [314]. TNF also cleaves vascular endothelial (VE)-cadherin [318], an endothelium specific adhesion molecule important in maintaining

endothelial tight junctions, whose cleavage could result in increased permeability, interstitial edema, and hemorrhage [316].

In vitro, IL-6 and TNF upregulated oxidized LDL uptake, transforming macrophages into foam cells, a process significantly augmented by the serum of RA patients [319]. These processes were attenuated with inhibition of IL-6 or TNF, supporting the beneficial effect of these therapies on the reduction of atherosclerosis risk [319].

An increase in the carotid intima-media thickness (cIMT), an indicator of subclinical atherosclerosis, have been described since early RA [320,321]. Nevertheless, RA has also been associated with CV disease, despite of cIMT increase, in association with maladaptive outward carotid arterial remodeling [2,322].

The inflammatory environment that occurs in RA induces bone remodeling disturbances, contributing not only to bone erosions but also to the development of secondary OP. As in physiological conditions, RANK/RANKL/OPG system is a major player in bone resorption in RA [121,323,324]. On the other hand, TNF produced by inflammatory cells induces the up regulation of sFRP and DKK proteins inhibiting the Wnt signaling pathway and compromising OB function and matrix mineralization [325,326]. The sequence and quantity of bone formation is controlled both by the temporal regulation and the level of expression of DKK1 [173]. TNF was identified as the key inducer of DKK1 both in animal models and in human RA [325]. Indeed, both in vitro and in vivo, TNF increases DKK1 expression, blocking OB differentiation, and inhibition of DKK1 not only rescued OB formation but also neutralizes TNF-mediated SOST expression in fully differentiated OBs [327]. TNF increased the expression of inhibitors of the Wnt signaling pathway such as DKK1 and SOST and could precipitate a destructive combination of low bone formation and high bone resorption [328]. Thus, OB differentiation and function are also abnormal in this disease, leading to the uncoupling of bone formation and bone resorption.

Systemic lupus erythematosus bones and vessels

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by multi-organ involvement and by the production of autoantibodies directed against a variety of nuclear and cytoplasmic antigens [329], and by an anomalous activation of the interferon pathway. SLE predominantly affects women of childbearing age, with a peak incidence in the 3rd decade of life, and 3/4 of the cases are diagnosed between 16 and 49 years of age [330]. There are large variations in the prevalence and natural history of SLE depending on the

geographical areas and ethnic groups studied [331–334]. These differences may be due to the genetic diversity of the populations concerned, but also to environmental and socio-economic factors. In Portugal, it has been reported that the prevalence of SLE is 0.1% (95% CI 0.1%-0.2%) and it is more frequent in women than in men [271].

The great diversity of phenotypic manifestations and the evolution by outbreaks, in which periods of exacerbation intersect with periods of remission, are characteristic of this disease.

Some antibodies are relevant to diagnosis, whereas others are associated with prognostic features or disease activity status [335,336]. Antibodies against double-stranded DNA (anti-dsDNA) and Smith antigen (anti-Sm) are considered very specific for SLE diagnosis, and both are part of the immunologic classification criteria for this disease [337]. Furthermore, high levels of anti-dsDNA are associated with higher disease activity in SLE [338,339]. The severity of the disease is determined by the intensity of the inflammatory response. Persistent inflammation, not effectively controlled, leads to irreversible damage that accumulates over the course of the disease's progression.

The evaluation of inflammatory activity and irreversible damage are features that should be part of the regular monitoring of these patients [340], and there are several instruments available for this purpose. Among the valid, reproducible and sensitive indexes for measuring lupus activity [341], the most used are systemic lupus erythematosus disease activity index (SLEDAI), for its ease of use [342] and British Isles Lupus Assessment Group (BILAG) for being more informative [343]. SLEDAI is a global index developed, validated and introduced in the clinic in 1985 and accounts for the presence of clinical and laboratory manifestations of lupus activity in the previous 10 days [342]. In 2002 a revised and validated version was introduced, SLEDAI-2K [344]. The score obtained by this instrument can vary between 0 - 105 and the disease is considered active if the result is ≥4 [345]. To evaluate the damage, a single device, the Systemic Lupus International Collaborating Clinics / ACR Damage Index (SLICC-DI), is available [346]. This index quantifies permanent and irreversible lesions accumulated since diagnosis, regardless of whether they result from inflammatory activity, treatment, co-morbidity or a combination of these factors. The SLICC-DI score is always increasing and a higher score reflects a poor quality of life [347] and a higher risk of death [348,349]. Several predictors of damage have been identified, including advanced age, some clinical and laboratory characteristics, chronic steroids, the presence of co-morbidities and the existence of previous damage are predictive of greater damage in the future [350–352].

Among the various rheumatic diseases that contribute to a decrease in life expectancy, SLE is the one in which the adjusted mortality rate is highest, 2 to 5 times higher than expected for the age group and for sex [284,353,354]. It is among the younger women with recent illness that the

highest mortality rate is found [353]. CV disease stands out among the causes of death. The contribution of CV pathology to late peak mortality remains constant [354–356]. Despite the overall increase in the life expectancy of SLE patients, CV mortality has remained unchanged over years [357]. On the basis of excess deaths from this cause is accelerated atherosclerosis and thrombotic phenomena [358]. Non-fatal CV events are also increased in SLE, with a prevalence ranging from 1.8% to 27%, depending on the duration of the disease [359]. SLE patients present not only an increased risk of fatal and non-fatal CV events but also of subclinical atherosclerosis and female gender and premenopausal status do not provide protection against atherosclerotic disease. The prevalence of carotid atherosclerotic plaques ranged from 17% to 32% in patients without previous CV events [360,361].

SLE patients also have a high morbidity related to bone loss and associated increased fracture risk [362–364]. Disease flares and cumulative organ damage are associated with bone loss [364]. OP was found in 10-68% [364] and osteopenia in 25-75% of SLE patients [363,364]. Multiple studies have demonstrated decreased BMD levels in SLE patients, both at diagnosis and at severe disease [362,364,365], as well as an increased fracture risk in both men and women [362]. However, BMD does not necessarily correlate with fracture risk, as 4-30% may develop fragility fractures despite normal BMD [364]. Risk factors contributing to bone loss in SLE include inflammation, premature menopause, physical inactivity, sun avoidance (with the consequent decrease in vitamin D levels) and treatment with corticosteroids [365,366]. Additionally, patients with SLE share inherent OP risk factors with healthy age-matched counterparts, including sex, postmenopausal status, age, family history, lifestyle, and low body mass index (BMI) [364,365].

The etiology of SLE is multifactorial, influenced by genetics, systemic inflammation, serological, metabolic, and hormonal factors, lifestyle choices and changes in body composition [363]. The cause of SLE remains unknown, but it is generally accepted that lupus results from an interaction between genetic and environmental factors, leading to complex changes in the innate and adaptive immune system.

Epigenetic alterations, dependent on external stimuli, are fundamental for the regulation of gene expression and establish a bridge between genetic and environmental factors [367].

In SLE, oligoclonal expansion of T lymphocytes, defects in signaling and altered production of various cytokines (↓IL-2, ↑INF-α, ↑IL-6, ↑IL-10, ↓TGF-ß) were documented [329,368]. Additionally, hyper-reactive B lymphocytes play a key pathogenic role through the production of numerous antibodies against nuclear antigens, but also to the cytoplasm and the cell surface. However, the role of B cells in SLE goes beyond the production of autoantibodies, and it is possible to induce lupus in animal models incapable of producing immunoglobulins, provided autoimmune B cells were present [369].

Elevated levels of IL-6 have been reported to abrogate DNA methylation in B cells, promoting the activation and expansion of autoreactive B cells in SLE patients [329]. It has also been described that IL-17 increases the survival and proliferation of B cells and their transformation into antibody-secreting cells [329].

The pathological features characteristic of lupus includes the deposition of immunocomplexes, inflammation and vascular injury. The autoantibodies are responsible for the formation of antibody-antigen complexes that mediate the inflammatory process leading to dysfunction and injury of various organs. The deposition of immune complexes in tissues leads to complement activation, increased production of type I interferon and other cytokines fundamental to the activation of T and B lymphocytes. In SLE patients not only there is an increased production of immunocomplexes, as its removal is slower [370].

The pathologic vascular changes in SLE are characterized first by immune complex deposition, subsequent complement activation, swelling, dystrophy and desquamation of ECs, plasmatic impregnation of the walls, fibrinoid necrosis, and infiltrative-proliferative cellular reaction with small vessel luminal thrombosis followed by the development of sclerosis and premature atherosclerosis. With chronicity, all these pathologic changes result in marked atherosclerosis, even in young individuals [371].

Also, SLE patients have an altered lipid profile, influenced by several factors including elevated levels of the inflammatory cytokines IL-6 and TNF, with increased very-low-density lipoprotein (VLDL) and triglycerides but reduced high-density lipoprotein (HDL), promoting the oxidation of LDL and the development of atherosclerosis [372].

SLE patients have a reduced number [373] or an impaired function [374] of endothelial progenitor cells (EPCs), which play a role in endothelial repair and angiogenesis, independently of the presence of subclinical atherosclerosis [373]. Metabolic syndrome, very frequent in these patients and strongly related to inflammation, was reported to contribute to the reduction of circulating EPCs and increased arterial stiffness [375].

The pathophysiology of OP in patients with SLE is likely a combination of several factors, including traditional OP risk factors, inflammation, metabolic factors (such as vitamin D deficiency), and corticosteroids therapy [376,377].

Increased cytokine levels (e.g. TNF, IL-1β and RANKL) in bone tissue due to inflammation influence differentiation and activity of OBs, osteocytes and OCs, leading to disproportionate remodeling by increased bone resorption with reduced formation and thus net bone loss [366,378].

Glucocorticoids, extensively used for the treatment of SLE disease flares and complications, lead to significant bone loss through inhibition of the differentiation of OBs, impair the

production of some anti-resorptive cytokines (e.g. OPG) and increase bone resorptive activity [379]. Systemic use of glucocorticoids increases the risk of fracture, either due to decreased BMD, changes in bone structure or a combination of these [378,380].

Animal models of atherosclerosis

Experimental animal models are a valuable tool for providing information on etiology, pathophysiology, and complications of a disease and on the efficacy and mechanism of action of various drugs and compounds used in treatment [381,382]. An animal model is easily manageable, as compounding effects of dietary and environmental factors can be controlled. Blood vessels and cardiac tissue samples can be taken for detailed experimental and biomolecular examination [381]. The first evidence that experimental atherosclerosis could be induced in animals came into view at the beginning of the 20th century by Alexander Ignatowski; since then, numerous animal models have been used for understanding the mechanisms involved in both induction and regression of atherosclerotic lesions [381,383].

Despite its limitations, the mouse remains the favored species for atherosclerosis investigation. The two most frequently used models of mouse atherosclerosis are the $ApoE^{-/-}$ model (the first mouse gene successfully deleted for atherosclerosis research in 1992 and still the most widely used) and the $LDLR^{-/-}$ model [382–384].

The ApoE knockout mice is a very well established model to study atherosclerosis progression. The loss of the gene encoding apolipoprotein E (ApoE), a glycoprotein synthesized mainly in the liver and brain that serves as a structural component of VLDL particles, loses their function as a ligand for LDL-receptors, predisposing mice to develop atherosclerotic lesions resembling those seen in humans [385].

ApoE-deficient mice are generated by targeting and inactivating the apoE gene, which is essential for the transport and metabolism of lipids, leading to lipid accumulation and atherosclerotic plaque formation.

These ApoE knockout ($ApoE^{-/-}$) mice tend to develop spontaneously and in a short time [386] a full range of atherosclerotic lesions distributed throughout the arterial tree, with progression from fatty streaks to fibrous plaques [387,388]. Although mice do not experience plaque rupture, contrary to humans where this is a very common complication of atherosclerosis, they are

hypercholesterolemic due to probable delayed clearance of large atherogenic particles from the circulation. Female ApoE$^{-/-}$mice develop atherosclerotic lesions more rapidly [389] and with higher mean lesion area [390] than their male counterparts.

Because this pathology can be exacerbated with a diet rich in fat and cholesterol, with appearance of fatty streaks in the proximal aorta at 3 months of age [387], this model is adequate to study atherosclerosis progression [388].

IL-1β exerts an atherogenic action in this animal model by enhancing the expression of VCAM-1 and MCP-1 in the aorta, which possibly increases the recruitment of monocytes/macrophages to the intima [391]. Additionally, it has been reported that ApoE$^{-/-}$ mice suffer a decrease on bone mass when animals get older [392], but also when they are fed with a high fat diet [393,394], possibly by blocking the differentiation of OB progenitor cells [395]. Interestingly, this process has been suggested as being associated to changes in inflammatory cytokines [394] but also to changes in genes associated with both bone resorption and formation [396]. Nevertheless, reduced bone mass has also been observed in C57BL/6 mice [395] and other animals [397–399] given a high-fat diet.

There are, however, some studies that have reported that ApoE$^{-/-}$ mice are characterized by high bone mass due to an increased bone formation rate [400,401]. Nevertheless, Bartelt et al. [400] have shown that high-fat diet induced a reduction of bone mass in ApoE$^{-/-}$ mice but not in the wildtype.

AIMS

Several epidemiological studies have shown that individuals with CV disease have a higher risk of fragility fractures. On the other hand, individuals with low BMD have more CV events and deaths. These two diseases, often present in the same individual, are responsible for high rates of morbidity and mortality and share common risk factors. Also, the inflammatory process has been implicated in the pathogenesis of both diseases, particularly in patients with immune-mediated rheumatic diseases. We thus hypothesize that bone and vascular disturbances share common pathophysiological mechanisms and that a connection between the progression of both conditions exists.

The present work aims to understand the interaction between vessels and bones in the context of rheumatic inflammatory diseases through the study of the effect of inflammation on the tissues.

The specific aims of this study were:

I. To evaluate how, in inflammatory rheumatic diseases, the vessels are disturbed, leading to vascular alterations and CV disease;
II. To evaluate how bone metabolism biomarkers are affected by inflammatory rheumatic diseases;
III. To understand the role of inflammation as a common contributor to the interplay between bones and vessels, in the context of health and illness.

MATERIAL AND METHODS

To achieve the first aim, a convenience cohort of patients with SLE and RA women fulfilling the ACR classification criteria and without previous CV events were consecutively recruited between May 2007 and October 2010. A control group of women without systemic inflammatory diseases was also recruited. Demographic data, disease characteristics, current medication, and CV risk profile including blood pressure, serum lipids, fasting glycemia, smoking habits, and BMI were obtained. Cytokines and soluble vascular biomarkers were determined using commercial enzyme-linked immunosorbent assays (ELISA); endothelial function was assessed by peripheral artery tonometry (PAT), a noninvasive operator-independent method that evaluates changes in pulse wave amplitude before and after reactive hyperemia; high-definition carotid ultrasonography was assessed for a subgroup of RA patients, with cIMT measured in the common carotid artery and the identification of carotid atherosclerotic plaques.

Patients with RA were reassessed after 5 years of follow-up and the incidence of CV events identified (acute myocardial infarction, unstable angina and coronary revascularization through percutaneous coronary intervention or coronary artery bypass grafting, stroke, transient ischemic attack, peripheral artery disease and death due to any of these events.).

For the second aim, SLE patients were studied and serum measurements of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), lipids (total cholesterol, HDL, LDL and tryglicerides), anti-dsDNA and anti-Sm antibodies, and sRANKL and OPG were performed.

For the third aim, ApoE deficient female mice were used, and as a control group we used C57BL/6 strain female mice. All groups were fed a high-fat diet (atherogenic diet) and mice were euthanized with CO_2 at different time-points: 8, 16, 20, 24 and 28 weeks old. Blood (for bone markers quantification by ELISA), the aorta (for either lesions quantification with oil red O staining or gene expression studies of inflammation and bone proteins), and bones (femurs and tibias for three-point bending tests and L3-L4 vertebras for histomorphometry analysis) were collected from all mice.

Furthermore, a sample of bone from the iliac crest and a section of the abdominal aorta artery were collected from 45 cadaver donors at the time of organ collection for transplantation, between April 2013 and September 2015. In addition, atherosclerotic plaques were collected from 139 patients submitted to carotid endarterectomy surgery between May 2012 and May 2015; all patients performed a DXA and a structured protocol was applied to all patients for recording demographic data, CV risk factors, history of previous factures, personal and family history of OP, other comorbidities, lifestyle and nutritional habits, and past and current

medication. Fasting blood samples were collected from both donors and patients for cytokines and bone markers quantification. RNA was extracted from the biological samples for gene expression determination of bone remodeling and inflammatory proteins. The atherosclerotic plaques were evaluated by histological and immunohistochemical techniques for calcium, inflammatory cells and adiponectin content.

Materials and methods are further detailed in the publications inserted in the Results section.

RESULTS

In agreement with the Decreto-Lei 388/70, art. 8º, the results presented and discussed in this thesis were published or submitted for publication in the following scientific peer-reviewed journals:

I. Santos MJ, **Carmona-Fernandes D**, Canhão H, Canas da Silva J, Fonseca JE, Gil V. *Early vascular alterations in SLE and RA patients--a step towards understanding the associated cardiovascular risk*. PLoS One. 2012; 7(9): e44668.

II. Castro AM, **Carmona-Fernandes D**, Rodrigues AM, Pedro LM, Santos MJ, Canhão H, Fonseca JE. *Incidence and predictors of cardiovascular events in a cohort of patients with rheumatoid arthritis*. Acta Reumatol Port. 2016 Jul-Sep;41(3):213-219.

III. **Carmona-Fernandes D**, Santos MJ, Perpétuo IP, Fonseca JE, Canhão H. *Soluble receptor activator of nuclear factor κB ligand/osteoprotegerin ratio is increased in systemic lupus erythematosus patients*. Arthritis Res Ther. 2011;13(5):R175.

IV. **Carmona-Fernandes D**, Casimiro RI, Koskela A, Finnilä M, Santos MJ, Canhão H, Fonseca JE. *Bone disturbances and progression of atherosclerosis in ApoE knockout mice*. (Under submission)

V. **Carmona-Fernandes D**, Leonardo N, Casimiro RI, Castro A, Barreira S, Santos P, Fernandes AN, Cortes-Figueiredo F, Gonçalves C, Cruz R, Fernandes M, Ivo M, Pedro LM, Canhão H, Fonseca JE, Santos MJ. *Atherosclerosis and Bone Disease in Humans – results from cadaver donors and endarterectomized patients*. (Under submission)

I. Early vascular alterations in SLE and RA patients - a step towards understanding the associated cardiovascular risk

Santos MJ, **Carmona-Fernandes D**, Canhão H, Canas da Silva J, Fonseca JE, Gil V.; PLoS One. 2012; 7(9):e44668.

Carmona-Fernandes D carried out the experiments and contributed to reagents/materials/analysis tools.

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Early Vascular Alterations in SLE and RA Patients—A Step towards Understanding the Associated Cardiovascular Risk

Maria José Santos[1,2]*, Diana Carmona-Fernandes[1], Helena Canhão[1,3], José Canas da Silva[2], João Eurico Fonseca[1,3], Victor Gil[4]

1 Rheumatology Research Unit, Instituto de Medicina Molecular da Faculdade de Medicina de Lisboa, Lisbon, Portugal, **2** Rheumatology Department, Hospital Garcia de Orta, Almada, Portugal, **3** Rheumatology and Metabolic Bone Diseases Department, Hospital de Santa Maria, Lisbon, Portugal, **4** Cardiology Department, Hospital Fernando Fonseca, Amadora, Portugal

Abstract

Accelerated atherosclerosis represents a major problem in both systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients, and endothelial damage is a key feature of atherogenesis. We aimed to assess early endothelial changes in SLE and RA female patients (127 SLE and 107 RA) without previous CV events. Biomarkers of endothelial cell activation (intercellular adhesion molecule-1 (sICAM-1), vascular cell adhesion molecule-1 (sVCAM-1), thrombomodulin (TM), and tissue factor (TF)) were measured and endothelial function was assessed using peripheral artery tonometry. Reactive hyperemia index (RHI), an indicator of microvascular reactivity, and augmentation index (AIx), a measure of arterial stiffness, were obtained. In addition, traditional CV risk factors, disease activity and medication were determined. Women with SLE displayed higher sICAM-1 and TM and lower TF levels than women with RA ($p = 0.001$, $p < 0.001$ and $p < 0.001$, respectively). These differences remained significant after controlling for CV risk factors and medication. Serum levels of vascular biomarkers were increased in active disease and a moderate correlation was observed between sVCAM-1 levels and lupus disease activity (rho = 0.246) and between TF levels and RA disease activity (rho = 0.301). Although RHI was similar across the groups, AIx was higher in lupus as compared to RA ($p = 0.04$). Also in active SLE, a trend towards poorer vasodilation was observed ($p = 0.06$). In conclusion, women with SLE and RA present with distinct patterns of endothelial cell activation biomarkers not explained by differences in traditional CV risk factors. Early vascular alterations are more pronounced in SLE which is in line with the higher CV risk of these patients.

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* E-mail: mjps@netvisao.pt

Introduction

Chronic systemic inflammation predisposes to accelerated atherosclerosis, a risk that is well known in systemic lupus erythematosus (SLE) and in rheumatoid arthritis (RA) patients [1]. Subclinical vascular lesions develop long before atherosclerosis becomes clinically evident, and they progress more rapidly in SLE [2] and RA [3] than in the general population. Traditional cardiovascular (CV) risk factors do not fully explain this enhanced risk, and the disease itself is considered an independent CV risk factor [1]. In addition, the potential contribution of genetic variants to the development of atherosclerosis in RA patients has been recently highlighted [4,5]. However, the reported magnitude of the CV risk is several times higher in SLE than in RA [6 9], and the reason for this divergence is still incompletely understood.

Endothelial damage is considered the first step in the pathogenesis of atherosclerosis. It correlates with disease progression and predicts CV events in the general population [10]. The importance of endothelial cells (ECs) for vascular health is highlighted by its crucial role in maintaining blood fluidity and in regulating vascular tonus and permeability. Under basal conditions ECs express molecules such as thrombomodulin (TM), which prevent platelet aggregation and the activation of the clotting cascade. Further platelet inhibition is achieved as a result of nitric oxide (NO) synthesis, a major vascular relaxant with anti-inflammatory and anti-proliferative properties. During the inflammatory process, ECs undergo changes characterized by enhanced expression of adhesion molecules, increased transendothelial permeability, and loss of antithrombotic properties [11]. Pro-inflammatory cytokines suppress TM expression and promote its cleavage and release into circulation [12]. In addition, they induce the expression of tissue factor (TF), a procoagulant molecule absent from the surface of the intact ECs [13], shifting the balance towards a prothrombotic state. Furthermore, damaged endothelium loses its ability to produce vasodilators, thus adding to the vascular injury. Endothelial dysfunction is potentially a reversible disorder. Indeed, in patients with active RA, the infusion of infliximab, a chimeric antibody against TNF, has been found to improve biomarkers of endothelial activation [14] and transiently ameliorate endothelial function[15].

In vivo, vascular function can be examined non-invasively by quantifying biomarkers of endothelial activation/damage, by measuring the ability of endothelium to release NO in response to various stimuli or by assessing arterial wall stiffness [16]. Previous data indicate impaired endothelial function both in SLE [17] and in RA patients [18] when compared to non-inflammatory controls. Nevertheless it is unclear whether the magnitude of early vascular changes is similar in these two diseases.

Given the clinical and pathophysiological particularities of SLE and RA, we hypothesize that endothelial function is differently disturbed in these two patient groups, which could explain the different CV risk. Thus, the major aim of our study was to compare endothelial cell function between SLE and RA as assessed by the measurement of soluble vascular biomarkers and by endothelial function testing, taking into account the presence of traditional CV risk factors and systemic inflammation.

Materials and Methods

Subjects

Consecutive SLE and RA women fulfilling the ACR classification criteria and free of clinically manifest CV disease were recruited from the rheumatology clinics of Hospital Garcia de Orta, Almada, and Hospital de Santa Maria, Lisbon, between April 2009 and October 2010. A control group of women without systemic inflammatory diseases was also recruited from the local community and evaluated in the same period. Participants were excluded if they were pregnant, breastfeeding, had impaired renal function (defined as serum creatinine >1.5 mg/dl), or had documented ischemic heart disease (previous infarction, revascularization surgery, angina, or heart failure), cerebrovascular disease (stroke or transient ischemic attack) or symptomatic peripheral artery disease. The study was approved by the Ethics Committee of both hospitals and was conducted in accordance with the principles stated in the Declaration of Helsinki. All participants gave written informed consent.

Clinical assessment

Demographic data, disease characteristics, current medication, and CV risk profile including blood pressure, serum lipids, fasting glycemia, smoking habits, and body mass index (BMI) were obtained. Patients were diagnosed with hypertension if the measured blood pressure was repeatedly $\geq$140/90 mm/Hg or if they used antihypertensive medication. The diagnosis of diabetes was made if fasting glucose level was $\geq$126 mg/dl, or if patients were under pharmacological treatment. Participants were classified as obese if BMI was ≥ 30 Kg/m^2. Disease activity was evaluated using the SLE Disease Activity Index 2000 (SLEDAI 2K), [19] and in RA patients 28 joints were examined for tenderness and swelling, and the 4 variable disease activity score (DAS28) was calculated using erythrocyte sedimentation rate [20]. Disease activity was stratified according to the cutoffs of each instrument [20,21]: remission (SLEDAI 2K = 0 for SLE or DAS28$<$2.6 for RA patients), low disease activity ($\geq$1 SLEDAI 2K$<$4, in the case of SLE, or $\geq$2.6 DAS28$\leq$3.2, in the case of RA), and active disease (SLEDAI 2K$\geq$4 or DAS28$>$3.2, for SLE and RA patients, respectively).

Fasting blood samples were obtained before any other procedures for measurement of glucose, uric acid, lipids (total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and triglycerides), inflammatory mediators (C-reactive protein (CRP) and fibrinogen) and soluble vascular biomarkers (sICAM-1, sVCAM-1, TM, and TF).

Quantification of soluble vascular biomarkers and cytokines

Measurements were performed using commercial enzyme-linked immunosorbent assay (ELISA) based methods according to the manufacturers' instructions. The Human sICAM-1 FlowCytomix Simplex Kit and the Human sVCAM-1 FlowCytomix Simplex Kit (Bender MedSystems GmbH, Vienna, Austria) were used for quantification of adhesion molecules, both using the FlowCytomix ^{TM} Technology. Serum levels of TM were measured using the Human Thrombomodulin ELISA Kit (Cell Sciences ®, Canton, MA, USA) and serum levels of TF were quantified using the AssayMax Human Tissue Factor ELISA kit (Assaypro, St Charles, Mo, USA).

Endothelial function tests

Endothelial function was assessed by peripheral artery tonometry (PAT). PAT is a noninvasive operator-independent method that evaluates changes in pulse wave amplitude before and after reactive hyperemia. The inter-day variability of this technique in our department is 11% (data not published). The exam was performed using the EndoPAT 2000 device (Itamar Medical Ltd, Cesarea, Israel) as described elsewhere [22] and by assessors blinded to the clinical diagnosis. Briefly, patients were placed in a quiet room, in supine position, with a specially designed finger probe on the index finger of each hand, and a pressure cuff placed on one arm. Patients were recommended to refrain from smoking and drinking coffee or tea during the previous 24 hours and not to eat for at least 6 hours preceding the exam. PAT was continuously measured during a 10-minute baseline period, for 5 minutes after the pressure cuff was inflated to suprasystolic pressure and for 10 additional minutes following the release of upper arm occlusion. Pressure changes reflecting pulse amplitude were transmitted to a computer and reactive hyperemia index (RHI) was calculated as the ratio of PAT signal amplitude after cuff deflation divided by the amplitude of baseline signal, adjusted for fluctuations in the magnitude of the signal in the contralateral finger [22]. Augmentation index (AIx) was calculated from the mean PAT waveform of the baseline period dividing the amplitude of the second systolic peak by the difference between the second and the first peak.

Statistical analysis

Continuous variables are expressed as means with standard deviations and categorical variables as the number of affected individuals and proportion of the total. Bivariate comparisons of SLE and RA patients were made using Student T-tests, Mann-Whitney, Kruskal-Wallis or χ^2 tests, as appropriate.

The levels of vascular biomarkers, as well as RHI and AIx, were compared between SLE and RA patients first as crude means using the Mann Whitney test, followed by analysis of covariance (ANCOVA) to adjust for significant and clinically relevant baseline covariates. Likewise, in order to assess the effect of disease activity on vascular biomarkers, RHI and AIx, comparisons between remission and active disease were performed.

Correlation between disease activity and endothelial cell function was studied separately in SLE and RA using Spearman correlation coefficient and partial correlations to control for age, disease duration, cardiovascular risk factors and medication.

Statistical analysis was performed assuming a 5% significance level and using SPSS 17 for Windows.

Results

In total 127 women with SLE and 107 with RA, were included in the study. A control group of 124 women, mean age 46.9±13.7 years, 98% Caucasian, and 52% postmenopausal was also evaluated as reference. Demographic and clinical characteristics of SLE and RA patients are shown in Table 1. SLE women were younger and had shorter disease duration (8.4±6.5 years) compared to women with RA (10.7±7.3 years, p = 0.01). All lupus patients were ANA positive and 89% of RA patients were positive either for IgM RF or for anti-citrullinated protein antibodies. The use of antimalarials and aspirin was more common in lupus, while more RA patients received methotrexate. Serum concentration of fibrinogen was higher in SLE (SLE 326±147 mg/dl vs RA 276±101 mg/dl; p = 0.01), but CRP levels were similar in both groups (SLE 1.16±3.2 mg/dl vs 1.06±2.5 mg/dl; p = 0.75).

Vascular biomarkers and endothelial function as assessed by PAT

A distinct pattern of soluble ECs biomarkers was identified in SLE and in RA. While sICAM-1 and TM levels were significantly higher, TF was lower in lupus than in RA patients (Figure 1). Differences in sICAM-1, TM and TF remained significant after adjustment for covariates (Table 2).

Reactive hyperemia was similar in SLE (RHI = 2.135±0.686), in RA (RHI = 2.194±0.810) and in the control population (2.090±0.579), while AIx was significantly higher in SLE as compared to RA (16% vs 11%; p = 0.04), indicating increased arterial wall stiffness in these patients. This increase remained statistically significant after controlling for differences in baseline characteristics (Table 2).

Table 1. Demographic and clinical characteristics of SLE and RA women.

	SLE (n = 127)	RA (n = 107)	p value
Demographic data			
Age, years	43.9 (13.9)	50.2 (14.1)	0.01
Education, years	8.9 (4.8)	8.3 (5.2	ns
Caucasians, n (%)	110 (87)	95 (89)	ns
Menopause, n (%)	60 (47)	62 (58)	ns
Traditional CV risk factors			
Current smoker, n (%)	18 (14)	17 (16)	ns
Hypertension, n (%)	51 (40)	37 (35)	ns
Total cholesterol, mg/dl	189.3 (42.8)	204.0 (33.5)	0.005
HDL cholesterol, mg/dl	56.6 (16.1)	63.9 (18.5)	0.002
LDL cholesterol, mg/dl	113.9 (36.6)	123.4 (28.5)	0.04
Triglycerides, mg/dl	125.7 (88.2)	103.1 (42.8)	0.01
Diabetes, n (%)	9 (7)	5 (5)	ns
Obesity, n (%)	33 (26)	33 (31)	ns
Medication			
Antihypertensive, n (%)	51 (40)	32 (30)	ns
Lipid lowering, n (%)	31 (24)	17 (16)	ns
Aspirin, n (%)	28 (22)	6 (6)	<0.001
Hydroxychloroquine, n (%)	97 (74)	20 (19)	<0.001
Methotrexate, n (%)	12 (10)	85 (82)	<0.001
Prednisolone, mg/day	7.8 (10.9)	3.2 (3.5)	0.006

Results are presented as means (SD) or number of affected individuals and (%). SLE – systemic lupus erythematosus; RA – rheumatoid arthritis; CV-cardiovascular; HDL – high density lipoprotein; LDL – low density lipoprotein, ns – non significant.

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Disease activity and endothelial function

Patients presented a broad range of disease activity. The mean SLEDAI 2K was 3.46±4.5 (range 0 to 21) and the mean DAS28 was 4.19±1.4 (range 1.70 to 7.54). Disease was in remission in 41% of SLE and in 17% of RA cases. 39% of SLE patients presented moderate/high active disease defined as a SLEDAI 2K≥4, and 72% of RA had moderate/high active disease according to the DAS28 definition. Except for prednisolone dosage, which was significantly higher in active SLE and active RA than in remission, demographic characteristics, CV risk profile and medication was comparable in quiescent and active disease.

Overall, serum levels of vascular biomarkers were elevated when disease was active, being statistically significant the differences in sICAM-1, TM and TF levels between active and quiescent SLE and in sICAM-1 and TF levels between active RA and remission (Table 3).

sVCAM-1 showed a significant Spearman and partial correlation with lupus disease activity measured by the SLEDAI (rho 0.246 and 0.361; p = 0.007 and p<0.001 respectively) and TM levels correlated with DAS28 (rho 0.301 and 0.250; p = 0.002 and p = 0.005). In SLE patients there was also a significant correlation between sVCAM-1, TM, TF and ESR (rho 0.246, 0.323 and 0.263; p = 0.01, p = 0.001 and p = 0.01, respectively) and between serum TM levels and CRP (rho 0.315; p = 0.001). No significant correlation was found in RA patients between ESR or CRP and vascular biomarkers.

A trend toward lower RHI was observed in active SLE (1.806±0.16) as compared with remission (2.249±0.13; p = 0.06), but no significant correlation was observed between RHI, AIx and SLEDAI or DAS28.

Discussion

In this comparative study we found distinct patterns of soluble vascular biomarkers in SLE and in RA female patients free from clinically evident CV disease. Lupus patients presented higher serum sICAM-1 and TM levels, while TF was elevated in RA patients. These findings are relevant for understanding the pathophysiology of the increased CV risk in SLE and RA patients, as cell adhesion molecules may represent a link between inflammation and atherosclerosis. In fact, not only are VCAM-1 and ICAM-1 highly expressed on the endothelium overlaying atherosclerotic lesions [23,24], but an increased serum concentration of these molecules is also related to CV risk factors [25] and incident myocardial infarction [26]. In particular, high serum levels of ICAM-1 represent an independent risk factor for atherosclerosis and a predictor of future CV events [26,27]. In addition, we observed significantly increased levels of vascular biomarkers in active disease. These observations are in line with previous studies demonstrating that inflammatory mediators, including TNF, IL-6, interferon-gamma (INFγ) [28], IL-18 [29], but also MCP-1 and MIF [30], upregulate endothelial cell adhesion molecule expression. The fact that SLE patients exhibit higher sICAM-1 and also higher fibrinogen concentrations may be relevant in the initiation and progression of atherosclerosis. Indeed, ICAM-1 serves as a binding site for fibrinogen and promotes adhesion and transendothelial migration of leukocytes [31], an important early step in inflammatory vascular disease. We did not find any difference in VCAM-1 serum levels among the

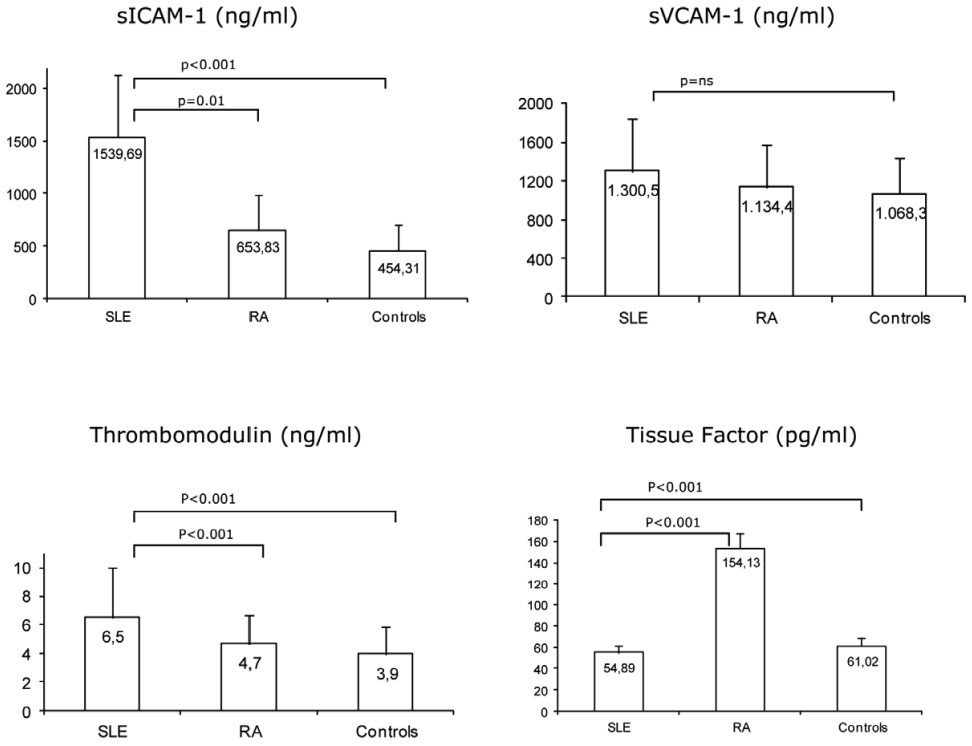

Figure 1. Serum concentrations of vascular biomarkers in SLE and RA patients and non-inflammatory controls. sICAM-1– soluble intercellular adhesion molecule; sVCAM-1 – soluble vascular cell adhesion molecule; RHI–reactive hyperemia index; Aix – augmentation index; SLE – systemic lupus erythematosus; RA – rheumatoid arthritis.
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studied groups. In animal models, VCAM-1 expression is considered a major early event in the atherosclerotic process [32], and increased sVCAM-1 levels have been reported in lupus nephritis [33]. However, in RA and SLE patients without renal or vascular disease, serum concentrations of VCAM-1 are similar to the control population and the relationship to atherosclerosis is uncertain [34,35]. Nevertheless, very recently sVCAM-1 was identified as an independent predictor of overall and cardiovascular mortality in SLE[36].

Table 2. Vascular biomarkers and results of PAT assessment in SLE and RA, after controlling for baseline covariates.

	Age adjusted			Adjusted for CV risk factors, disease duration and medication*		
	SLE (n = 127)	**RA (n = 107)**	**p value**	**SLE(n = 127)**	**RA(n = 107)**	**p value**
sICAM ng/ml	1436 (600)	778 (651)	0.01	1994(879)	398(898)	0.05
sVCAM, ng/ml	1313 (114)	1129 (125)	0.29	1330 (159)	1160(162)	0.53
TM, ng/ml	6.59 (0.27)	4.54 (0.29)	<0.001	6.47 (0.36)	4.58(0.37)	0.003
TF, pg/ml	56.3 (10.1)	152.6 (10.9)	<0.001	50.1 14.4)	157(14.7)	<0.001
RHI[§]	2.128 (0.08)	2.203 (0.09)	0.53	2.008(0.11)	2.309(0.12)	0.12
AIx[§]**, %**	17.9 (1.9)	9.5 (2)	0.003	20.3 (2.5)	8.3 (2.7)	0.007

Results are presented as estimated marginal means (SE).
*Adjusted for the following covariates: age, disease duration, total cholesterol, HDL, LDL, triglycerides, aspirin, hydroxychloroquine, methotrexate use, and prednisolone dose.
[§]RHI and AIx results refer to 87 women with SLE and 75 with RA.
sICAM-1 – soluble intercellular adhesion molecule; sVCAM-1 – soluble vascular cell adhesion molecule; TM – thrombomodulin; TF – tissue factor; RHI – reactive hyperemia index; AIx – augmentation index.
doi:10.1371/journal.pone.0044668.t002

Table 3. Vascular biomarkers and endothelial function in active disease and in remission.

	SLE (N = 127)			RA (N = 107)		
	Remission	Active	p	Remission	Active	P
sICAM-1	713.9 (147)	1952 (100)	0.04	527 (10.2)	668 (4.7)	0.01
sVCAM-1	1022 (69)	1419 (47)	0.02	1013 (94)	1171 (44)	0.15
TM	6.1 (0.5)	7.2 (0.4)	0.04	4.59 (0.2)	5.01 (0.4)	0.42
TF	54.1 (7.3)	58.6 (1.9)	0.08	126.6 (33)	160.4 (15)	0.03
RHI*	2.249 (0.13)	1.806 (0.16)	0.06	2.444 (0.21)	2.133 (0.10)	0.20
AIx*	14.4 (3.6)	18.5 (2.5)	0.03	12.2 (6.4)	10.8 (2.4)	0.79

Results are expressed as estimated marginal means (SE) adjusted for prednisolone dose.
*RHI and AIx results refer to 87 women with SLE and 75 with RA.
SLE – systemic lupus erythematosus; RA – rheumatoid arthritis; sICAM-1 – soluble inter-cellular adhesion molecule; sVCAM-1 – soluble vascular cell adhesion molecule; TM – thrombomodulin; TF – tissue factor; RHI – reactive hyperemia index; AIx – augmentation index.
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There is growing evidence supporting the relationship between inflammation and thrombotic complications of atherosclerosis (atherothrombosis). Interestingly, TM expression, a molecule with anti-coagulant properties, is reduced during the inflammatory process [12], and increased soluble TM levels probably indicate EC injury. Together with increased TF, which is an initiator of the extrinsic coagulation cascade, this environment may raise the thrombogenic activity of plasma and contribute to cardiovascular events. Higher levels of TF in RA patients as compared to SLE patients might be explained by the contribution of TNF to its expression [37]. Nevertheless, serum levels of adhesion molecules, TM, and TF may not accurately translate endothelial functional expression of these molecules, which is a limitation of our work.

A further effect of proinflammatory cytokines on EC is the inhibition of NO synthesis leading to endothelial dysfunction. In the general population, impaired endothelial function is a critical early step in the development of atherosclerosis [38] and predicts the progression of structural arterial disease independently of conventional CV risk factors [39,40]. However, studies of endothelial function in inflammatory rheumatic diseases depicted contradictory results [41 43], and the relevance of endothelial dysfunction for the progression of atherosclerosis in rheumatic diseases remains uncertain [44 46]. Similarly, the improvement following anti-rheumatic medication is not universally supported by the available literature [18]. Using PAT, we did not find any significant differences in RHI neither between patients and controls, nor between SLE and RA. RHI quantifies changes in pulse wave amplitude in response to reactive hyperemia, a measure of microvascular function. In the general population RHI is an independent predictor of adverse cardiac events [47], but its predictive value in rheumatic diseases has not been established. The fact that we have included only females without previous CV events and normal renal function (relatively low risk population) may in part account for the comparable RHI found in patients and controls. In fact, only in more active SLE cases did RHI show a reduction. The follow up of these patients will allow us to ascertain the predictive value of RHI measured by PAT for the development of CV event in SLE and RA patients.

Lupus patients presented higher AIx than RA patients and this difference remained significant after controlling for covariates. In apparently healthy subjects arterial stiffness is an independent predictor of coronary heart disease and stroke [48], but the predictive value of AIx in rheumatic diseases is unknown. Cardiovascular risk factors and disease related features contribute to arterial stiffening in SLE [49] and RA [50]. Shang et al found a correlation between carotid AIx and SLEDAI [51]. Increased arterial stiffness was also associated with RA disease activity in some, but not all, studies [18]. Increased AIx in SLE women probably indicates a worse vascular condition.

Taken together, our observations add to the evidence that the pathogenesis of atherosclerosis associated with inflammation may differ in SLE and RA. Additionally, we found more pronounced early vascular changes in lupus patients, and when the disease is active, which is in line with the higher risk for CV events documented in these patients.

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Author Contributions

Conceived and designed the experiments: MJS HC JEF VG. Performed the experiments: MJS DC-F. Analyzed the data: MJS HC JEF JCS VG. Contributed reagents/materials/analysis tools: MJS DC-F VG. Wrote the paper: MJS.

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II. Incidence and predictors of cardiovascular events in a cohort of patients with rheumatoid arthritis

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Carmona-Fernandes D carried out some laboratorial experiments and contributed to data analysis.

Incidence and predictors of cardiovascular events in a cohort of patients with rheumatoid arthritis

Castro AM[1], Carmona-Fernandes D[2], Rodrigues AM[3], Pedro LM[4], Santos MJ[5], Canhão H[1], Fonseca JE[1]

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ABSTRACT

Introduction: An excess in cardiovascular (CV) morbidity and mortality has been recognized in Rheumatoid Arthritis (RA) patients when compared to the general population. Given the paucity of prospective data, our aim was to estimate the incidence of CV events and the contribution of traditional CVD risk factors and RA-related parameters to future events.

Methods: Incident fatal and non-fatal CV events (hospitalizations due to unstable angina, myocardial infarction, coronary artery revascularization procedures, stroke, or CV death) were assessed in a prospective cohort of RA women followed since 2007 and without CV events at cohort entry. The presence of traditional CV risk factors, disease characteristics, medication, carotid ultrasound, and biomarkers of inflammation and endothelial activation were evaluated at baseline. Univariate Cox proportional hazard models were used to identify risk factors for CV events.

Results: Among 106 women followed over 565 patient-years we identified 4 CV events (1 fatal stroke, 2 myocardial infarction and 1 unstable angina), which contributed to an incidence rate of 7 per 1000 person-years (95%CI 2.0- 13.9). Patients who developed CV events were older, but the distribution of other traditional CV risk factors was otherwise similar in both groups. Also, corticosteroid dosage and proportion of patients with carotid atherosclerotic plaques was higher in those with CV events. Erythrocyte sedimentation rate (ESR) (HR 1.036; 95%CI 1.005-1.067) and soluble intercellular adhesion molecule-1 (sICAM-1) serum levels (HR 1.002; 95%CI 1.000-1.003) significantly contributed to CV events. These results remained significant after adjusting for patients' age.

Conclusion: We found an incidence of cardiovascular events in women with RA of 7 per 1000 patent-years. This value is similar to that found in other Portuguese cohort of RA patients[1] and much higher than the incidence reported for the general Portuguese population. Markers of inflammation and endothelial activation contributed significantly to CV events, but the limited number of events prevents further analysis.

Keywords: Cardiovascular risk; Rheumatoid arthritis; Carotid intima-media thickness

1. Serviço de Reumatologia e doenças ósseas metabólicas, Hospital de Santa Maria, CHLN
2. Instituto de Medicina Molecular, Unidade de Investigação em Reumatologia
3. Departamento de Reumatologia, Hospital de Santo Espírito de Angra do Heroísmo E.P.E.R, Terceira, Açores
4. Serviço de Cirurgia Vascular, Hospital de Santa Maria, CHLN
5. Serviço de Reumatologia, Hospital Garcia de Orta

INTRODUCTION

Cardiovascular disease (CVD) is a major public health problem and the leading cause of death in industrialized nations. In Portugal more than 37 000 deaths per year are attributed to CVD[1], representing almost 40% of all deaths.

Patients with inflammatory rheumatic diseases die prematurely, largely due to CVD. Atherosclerosis is now accepted to be a multifactorial process where inflammation plays a crucial role at each stage of the pathology. Atherosclerosis, the main determinant of cardiovascular (CV) morbidity and mortality, occurs prematurely in patients with inflammatory rheumatic diseases, such as Rheumatoid Arthritis (RA)[1]. These patients have almost a two-fold increased risk of CV events in comparison to the general population[2-3]. Traditional risk factors such as hypercholesterolemia, hypertension, diabetes, smoking and family history have long been identified as major contributors to the patho-

genesis of atherosclerotic lesions. However, these risk factors are present in only about 50% of RA patients with CV events[7] and do not fully explain this increased risk. Moreover, Framingham 10-year risk equation underestimates the true CV risk in these patients[6]. Increased CV risk is likely to be an effect of the disease *per se*, reinforced by the presence of traditional CV risk factors[3-5]. Therefore, the search for additional mechanisms linking RA to CV disease is relevant.

Data deriving from cross-sectional studies have shown that RA patients have evidence of advanced preclinical carotid atherosclerosis compared to healthy controls [8] to a magnitude similar to that observed in patients with diabetes mellitus (DM)[9]. High-resolution B-mode ultrasonography of the carotid artery provides a non-invasive, valid and reproducible method for identifying atherosclerotic plaques, which reflect prevalent, clinical or preclinical CV disease and may predict future CV events[10]. Notably, in RA patients without traditional CV risk factors or events, an increased intima-media thickness (IMT) of the common carotid artery and evidence of focal plaques were predictive of incident CV events[11,12]

Given the paucity of prospective data, the extent to which traditional CV risk factors and RA-related parameters (inflammatory burden, activity and/or remission and treatment modalities) interact and/or contribute to atherosclerosis acceleration remains inconclusive[13]. According to a recent study, both traditional CV risk factors and markers of RA severity at baseline contribute to models predicting CV events in the subsequent 22 months[14].

In the present work, we re-evaluated at 5 years a prospective cohort of 106 women with RA, that were recruited between 2007 and 2008 for a cross sectional study of subclinical atherosclerosis[3]. At cohort entry none had clinically apparent CV disease or previous CV events. Baseline assessment included demographic and clinical characteristics, evaluation of traditional CV risk factors, biomarkers of inflammation and endothelial activation and carotid artery ultrasound.

AIMS

1. To estimate the incidence rate of CV events in women with RA
2. To identify baseline predictors of future CV events in RA.

MATERIAL AND METHODS

STUDY POPULATION AND DESIGN

To address the objectives mentioned above, we used a unique prospective cohort comprising 106 women who met the American College of Rheumatology (ACR) classification criteria for RA, without previous CV events at the time of inclusion in the study. At baseline a clinical and laboratorial evaluation of patients was performed. A carotid ultrasound to measure the intima media thickness (IMT) was performed in 61 patients. Patients were prospectively followed up for approximately 5 years and incident CV events identified. Additionally, we performed a reassessment of patient charts to identify the possible occurrence of CV events over that period. We defined as CV events acute myocardial infarction, unstable angina and coronary revascularization through percutaneous coronary intervention or coronary artery bypass grafting, stroke, transient ischemic attack, peripheral artery disease and death due to any of these events.

The subjects provided their informed consent. The study was conducted according to the Declaration of Helsinki and was approved by the Ethics Committee of Hospital Garcia de Orta.

EVALUATION AND MANAGEMENT OF RA PATIENTS

The following variables were assessed at baseline:

RA MANIFESTATIONS

A physician assessed patients for tenderness, swelling or deformity in 28 joints and for subcutaneous nodules, erosions, pulmonary fibrosis, *sicca* syndrome, serositis, amyloidosis, vasculitis and episcleritis. We used the 28-joint count and the erythrocyte sedimentation rate (ESR) to calculate the disease activity score (DAS 28) and Health Assessment Questionnaire (HAQ) to assess functional status.

CV RISK FACTORS ASSESSMENT

Hypertension was defined by the use of antihypertensive medications, diastolic blood pressure >= 90 mmHg or systolic blood pressure >= 140 mmHg; Diabetes Mellitus by the use of antidiabetic medications or fasting blood sugar >=126 mg/dL; hypercholesterolemia by the use of lipid lowering medications or fasting plasma cholesterol >=190 mg/dl or low-density lipoprotein (LDL) cholesterol □130 mg/dl. Patients were considered current smokers if they smoked at

baseline and former smokers if they had quit. We defined obesity as a body mass index > 30 Kg/m^2.

Anti-rheumatic medication

The use of methotrexate, biologic agents and corticosteroids was registered. In the case of corticosteroids starting date, baseline and cumulative dose were also recorded.

Laboratory studies

Laboratory tests [erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), anti-cyclic citrullinated peptides (ACPA), cholesterol, low density proteins (LDL) and C-reactive protein (CPR)] were performed at baseline. Cytokines, biomarkers of endothelial cell activation and adipokines, such as OPG (osteoprotegerin), sRANKL (receptor activator of nuclear factor (NF)-kB ligand), TNF (tumor necrosis factor), tissue factor, thrombomodulin, sVCAM (soluble vascular cell adhesion protein 1), sICAM (soluble intracellular adhesion molecule 1), leptin, adiponectin, MIF (macrophage migration inhibitory factor), MCP-1 (monocyte chemoattractant protein 1), IL-10, IL-18 and insulin were also assayed.

Carotid ultrasound

Carotid intima-media thickness (cIMT) and the identification of carotid atherosclerotic plaques were assessed by B-mode ultrasonography in 61 patients at baseline.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20 software and significance was defined as $p<0.05$ throughout.

Patients with incident CV events over the follow-up period were compared with those who did not by using an independent sample t-test and the Mann-Whitney test (comparison of continuous variables as appropriate), as well as the χ^2 for comparison of categorical variables.

Univariate Cox regression proportional hazard models were used to identify traditional and non-traditional risk factors for a CV event.

Follow-up was calculated as the period between first and last observation or the date of occurrence of a first CV event.

RESULTS

INCIDENCE OF CV EVENTS OVER A PERIOD OF FIVE YEARS

Among 106 women followed up over 565 patient-years

TABLE I. BASELINE CHARACTERISTICS OF RA WOMEN. RESULTS ARE EXPRESSED AS MEANS ± STANDARD DEVIATION, EXCEPT OTHERWISE STATED

Demographics	
Age, years	49.87±13.96
Caucasian race, n (%)	94 (88,7%)
Cardiovascular rick factors	
Current tobacco use	18 (17%)
Hypertension, n (%)	35 (33%)
Dislipidemia	21 (19.8%)
Diabetes, n (%)	5 (4,7%)
Obesity (BMI>30)	33 (31.1%)
Atherosclerotic carotid plaques (n=61)	9 (14.8%)
Disease characteristics at baseline	
Disease duration at baseline, years	9.68±7.34
DAS 28	4,17±1.31
HAQ	1.11±0.71
RF positive, n (%)	79 (74.5%)
ACPA positive, n (%)	68 (64.2%)
Erosions, n (%)	60 (56.6%)
Rheumatoid nodules, n (%)	15 (14.2%)
Vasculitis, n (%)	2 (1.9%)
Amiloidosis	0
Episcleritis	0
Sicca syndrome, n (%)	16 (15.1%)
Serositis, n (%)	1 (0.9%)
Pulmonary fibrosis, n (%)	2 (1.9%)
Medication	
Corticosteroids use, n (%)	58 (54.7%)
Corticosteroids dose at baseline, mg	3,05±3,29
Cumulative dose of corticosteroids at baseline, mg	11139.45±11660.9
Methotrexate, n (%)	89 (84.0%)
Biologic therapy, n (%)	67 (63.2%)

RF: Rheumatoid Factor; ACPA: anti-cyclic citrullinated peptides; DAS: Disease Activity Score; HAQ: Health Assessment Questionnaire; BMI: Body Mass Index; CRP: C-Reactive Protein; ESR: Erythrocyte Sedimentation Rate; n: number

we identified 4 CV events, which contributed to an incidence rate of 7 per 1000 person-years (95%CI 2.0--13.9), a higher incidence when compared to the general Portuguese population.

A) Characteristics of the patients

All the 106 RA women included in the original study were re-accessed in December 2014. One death was registered and no patients were lost to the follow-up. At inception, the average age was 49.87 ± 13.96 years and most patients were Caucasian (88,7%). 74.5% (79) were RF-positive and 62,3% (66) had ACPA antibodies at baseline. 56.6% (60) had erosions on the X-rays, 14.2% (15) had rheumatoid nodules, 1.9% (2) had vasculitis, 15.1% (16) had sicca syndrome, 0.9% (1) had serositis and 1.9% (2) had pulmonary fibrosis.

Patients had an average disease duration > 9 years (9.68 ± 7.34 y) and active disease, with a mean DAS28 of 4.2 (4.17 ± 1.31). Furthermore, most patients were on a disease-modifying anti-rheumatic drug (DMARD) at baseline (89% methotrexate, 63.2% biological agents and 54.7% prednisolone). The average dose of prednisolone at baseline was 3.05 ± 3.29 mg.

Hypertension, dyslipidemia, current tobacco use, diabetes mellitus and obesity were present in 33% (71), 19.8% (21), 17% (18), 4.7% (5) and 31.1% (33) of patients, respectively. Carotid ultrasound was performed in 61 patients, of which 14.8% had at least one carotid plaque (CP) at baseline. According to ultrasound characteristics, three types of CP were identified in the cohort studied (types 1, 2 and 4). Type 4 CP (homogeneous and hyperechoic) were the most frequently founded.

B) Occurrence of CV events

During 565 patient-years we identified 4 CV events (1 fatal stroke, 2 myocardial infarction and 1 unstable angina).

Patients who developed CV events were older, but the distribution of other traditional CV risk factors was otherwise similar in both groups. Also, corticosteroid dose and the proportion of patients with carotid

TABLE II. BASELINE CHARACTERISTICS OF RA WOMEN WITH AND WITHOUT CV EVENTS

	With CV event (n=4)	Without CV event (n=102)	p value
Age, years	63.5 ± 4.2	49.3 ± 13.9	0.039
Caucasians, n (%)	3 (75)	91 (89.2)	0.673
RA duration, years	6.57 ± 7.13	9.80 ± 7.35	0.304
RF positive, n (%)	4 (100)	75 (73.5)	0.570
ACPA positive, n (%)	4 (100)	64 (62.7)	0.384
Mean DAS 28	4.34 ± 1.30	4.17 ± 1.31	0.676
Mean HAQ	1.5 ± 0.89	1.10 ± 0.70	0.356
Corticosteroids dose at baseline, mg	6.25 ± 4.79	2.93 ± 3.2	0.104
Smokers, n (%)	1 (25)	17 (16.7)	0.740
Hypertension, n (%)	2 (50)	69 (67.6)	0.597
Dyslipidemia, n (%)	1 (25)	20 (19.6)	0.796
Diabetes, n (%)	0	5 (4.9)	1.000
Obesity (BMI>30), n (%)	2 (50)	31 (30.4)	0.438
Atherosclerotic carotid plaques (n=61), n (%)	2 (50)	7 (12.3)	0.100
Type of atherosclerotic plaques	4	4, 2, 1	–
CRP, mg/dL	0.78 ± 0.91	1.0 ± 2.52	0.987
ESR, mm/h	66.25 ± 30.84	36.43 ± 23.01	0.049
sVCAM1, ng/ml	1034.92 ± 458.49	1147.20 ± 450.33	0.629
sICAM1, ng/ml	1226.69 ± 568.25	637.88 ± 418.07	0.006

Results are expressed as means ± standard deviation, except otherwise stated. RA: Rheumatoid Arthritis; RF: Rheumatoid Factor; ACPA: anti-cyclic citrullinated peptides; DAS: Disease Activity Score; HAQ: Health Assessment Questionnaire; BMI: Body Mass Index; CRP: C-Reactive Protein; ESR: Erythrocyte Sedimentation Rate; sVCAM1: Soluble Vascular Cell Adhesion Protein 1; sICAM1: Soluble intracellular adhesion molecule 1; n: number

atherosclerotic plaques was higher in those with CV events (Table I). Erythrocyte sedimentation rate (ESR) (HR 1.036; 95%CI 1.005-1.067) and soluble intercellular adhesion molecule-1 (sICAM-1) serum levels (HR 1.002; 95%CI 1.000-1.003) significantly contributed to CV events. These results remained significant after adjusting for patients' age.

Disease duration, DAS28 and treatment modalities used were not significantly different between groups. All other RA-related parameters, such as the presence of extra-articular manifestations, the presence of RF or ACPA antibodies were not significantly different between groups.

c) Factors associated with occurrence of CV events

We searched for parameters predictive of the occurrence of CV events over the time in our cohort. Comparison between groups showed that, independently of other covariates, patients with CV events were significantly older. ESR and levels of sICAM at the baseline seem to be independent predictors of the occurrence of CV events.

DISCUSSION

In this prospective study we examined factors contributing to the occurrence of CV events in RA patients. Patients with RA die prematurely of CVD and although the inflammatory component of RA is better controlled with the current standard of care, CV risk remains the double of that observed in individuals without RA[21]. As reported before, carotid atherosclerosis predicts future coronary events in RA, whereas those patients with carotid plaques (CP), multiple CV risk factors, active disease and high corticosteroids dose are at an even higher risk[16]. Additionally, not only the presence of CP but also certain characteristics of these plaques seem to be of great importance. A thin cap and less fibrous tissue inside atherosclerotic plaques make them more vulnerable to rupture leading to CV events. It has been proposed that the proinflammatory status in patients with RA causes more and/or more vulnerable plaques, explaining part of the elevated risk of CV events in RA[17].

We focused on the identification of predictive factors for the occurrence of CV events over 5 years in our cohort of RA women with moderate disease activity and we studied the differences between groups with CV events and those without. The main finding of our study was that ESR and the levels of sICAM seem to be independent predictors for the occurrence of CV events. It has been suggested that the proinflammatory status of RA patients is associated with a higher CV risk. Indeed, Semb *et al.* showed that lowering disease activity could lower CV risk in RA patients[22]. These results led to the assumption that active disease with higher cytokine levels is associated with higher CV risk. ICAM-1 has been implicated in the development of a large number of diseases. Many studies have hypothesized that increased production of cell adhesion molecules (CAMs) on the vascular endothelium plays a role in the development of arterial plaque, with the suggestion from both in vitro and in vivo studies that the CAM production is increased by dyslipidemia.

In addition there was a trend suggesting that higher doses of corticosteroids at baseline (6.25 ± 4.79 *vs.* 2.93 ± 3.2; p=0.103) and higher prevalence of carotid atherosclerotic plaques (50% *vs.* 12.2%; p=0.100) were associated with CV events. As reported by Evans *et al*, a higher cumulative corticosteroid dose confers a higher risk of acute coronary syndromes in RA patients[12]. More recently Giles *et al.*[15] reported prospective data suggesting that subclinical carotid atherosclerosis progression in RA patients is potentially modified detrimentally by cumulative prednisone exposure. Zampeli *et al.* demonstrated that formation of new atherosclerotic plaques depended on traditional CVD risk and corticosteroid use[16].

The presence of CP can be helpful in identifying RA patients at risk for future CV events, as some characteristics of these plaques seem to be of great importance. Different types of CP have been identified together with their specific CV risk. Reilly *et al*[25] reported that plaques could be characterized as homogeneous (types 3 and 4) or heterogeneous (types 1 and 2), with the latter being described as a combination of hyperechoic, isoechoic, and hypoechoic plaques. They also concluded that homogeneous plaques were correlated with a fibrous lesion on pathological examination and the heterogeneous plaques were correlated with the presence of intraplaque hemorrhage and ulceration. Previous studies[24,26] reported that heterogeneous plaques were more likely to result in plaque hemorrhage and adverse neurological events. Cerebrovascular events were mainly present in type 1 and type 2 lesions, whereas, type 3 and type 4 lesions were mainly asymptomatic. They also reported that type 1 and type 2 lesions were associated more frequently with intraplaque hemorrhage or ulceration.

Echodopler can detect carotid atherosclerosis before the occurrence of events, thus identifying patients at higher risk. However, it is not yet clear in which patients performing this exam in cost-effective.

In this cohort of patients with moderate disease activity, those with CV events had mainly type 4 CP at baseline, unlike to what would be expected since they developed CV events. However, we only have data from baseline and it would be interesting to re-evaluate these patients by carotid ultrasound to find what happened to CP characteristics. Together with traditional CV risk factors, the higher levels of sICAM and high dose of corticosteroids used may have influenced the increased vulnerability of the CP leading to the occurrence of CV events.

Our cohort was followed up over 565 patient-years and four CV events were identified, which contributed to an incidence rate of 7 per 1000 person-years (95%CI 2.0-13.9). Curiously, despite our sample size this value was similar to that previously found in another Portuguese RA patients cohort[18] and much higher than the estimated incidence for the general Portuguese population without RA (estimated annual incidence of stoke – 2.99 and ischemic heart disease – 0.9 per 1000 women)[19, 20].

The main limitation of this study arises from being based on a small sample size. Consequently, a low number of events were found, probably resulting in insufficient statistical power to detect small differences between the patients with and without CV events and thereby limiting the conclusions that we can address.

Another limitation of our study is we only included women in order to eliminate the variability associated with gender and select a more robust and consistent sample. Cardiovascular disease is one area in which there are significant gender differences. Estrogens are responsible for the most important differences between men and women with regard to the prevalence of cardiovascular disease. Loss of estrogen-related protection is possibly the main reason for the increased incidence of cardiovascular disease after menopause. Although gender is increasingly perceived as a key determinant in health and illness, gender studies are still lacking and we consider that our study could represent an added value in this regard.

The fact that a significant percentage of patients are under biological therapy may have impacted the incidence of CV events by reducing the inflammatory burden. Yet, and despite biological therapy, incident CV events in Portuguese RA women is still higher than the estimated incidence for the general Portuguese women. In order to clarify the role of biologics we need to analyze a larger sample.

Taken together, these data imply that tight control of RA disease activity combined with strict CV risk factor management deserves further study as a strategy to reduce atherosclerosis and the occurrence of CV events in these patients. Therefore, optimal RA management should aim to achieve not only sustained disease remission but also successful traditional CV risk reduction and this may have implications in terms of treatment regimen selection.

CONCLUSION

We found an incidence of cardiovascular events in women with RA of 7 per 1000 patent-years. This value is similar to that found by another Portuguese RA patients cohort[18] and higher than the incidence estimated for the general Portuguese women. Markers of inflammation and endothelial activation contributed significantly to CV events, though the limited number of events prevents further analysis.

CORRESPONDENCE TO
Alice Castro
Serviço de Reumatologia e Doenças Ósseas Metabólicas,
Hospital de Santa Maria
Av. Professor Egas Moniz, Lisboa
Portugal
E-mail: alicemcastro@gmail.com

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III. Soluble receptor activator of nuclear factor κB ligand/osteoprotegerin ratio is increased in systemic lupus erythematosus patients

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RESEARCH ARTICLE Open Access

Soluble receptor activator of nuclear factor κB ligand/osteoprotegerin ratio is increased in systemic lupus erythematosus patients

Diana Carmona-Fernandes[1†], Maria José Santos[1,2†], Inês Pedro Perpétuo[1], João Eurico Fonseca[1,3] and Helena Canhão[1,3*]

Abstract

Introduction: Systemic lupus erythematosus (SLE) patients have lower bone mineral density and increased fracture risk when compared with healthy individuals, due to distinct factors and mechanisms. Bone remodeling is a tightly orchestrated process dependent on several factors, including the balance between receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG).
Our aim was to assess serum OPG and soluble RANKL (sRANKL) levels as well as sRANKL/OPG ratio in female SLE patients and compare it with female controls.

Methods: We have evaluated 103 SLE patients and 114 healthy controls, all Caucasian females. All participants underwent a clinical and laboratory evaluation. sRANKL and OPG were quantified in serum by ELISA based methods. sRANKL, OPG and sRANKL/OPG ratio levels were compared between SLE patients and age, sex and race matched healthy controls. For SLE patients, a multivariate analysis was performed, to find the possible predictors of the changes in sRANKL, OPG and sRANKL/OPG ratio levels.

Results: Although sRANKL levels did not differ between the two groups, serum OPG was lower in SLE patients ($P < 0.001$). This led to an increased sRANKL/OPG ratio ($P = 0.010$) in the patients' group.
The multivariate analysis was performed considering age and other clinical and laboratorial potential confounders for these variations in the SLE patients group. We have showed that age ($P = 0.001$) and levels of anti-Sm antibodies ($P = 0.016$) were independent predictors of sRANKL/OPG ratio variations in SLE patients. No relationship with therapy or disease activity measured by SLEDAI2K was found.

Conclusions: These results are suggestive of increased osteoclastic stimuli driven by the SLE disease mechanisms.

Keywords: sRANKL, osteoprotegerin, systemic lupus erythematosus, osteoclastogenesis

Introduction

Systemic lupus erythematosus (SLE) is a chronic, multisystemic disease of unknown etiology characterized by chronic inflammation and damage to various organs and systems due to the production of autoreactive cells and antibodies [1-3].

SLE patients have lower bone mineral density (BMD) when compared with healthy individuals and are at increased risk of fracture [4-7]. Although corticosteroid exposure is a major contributor to bone loss in SLE [4,5,8]. disease activity and associated co-morbidities may contribute to this process [5,8]. In addition, vitamin D deficiency is a common finding among SLE patients, further contributing to impaired bone health [5].

Bone remodeling is a tightly orchestrated process in which osteoclasts attach to the bone surface and remove bone. After resorption, osteoblasts migrate into the lacunae and produce new bone, which then mineralizes [3,9]. Expression of the receptor activator of nuclear factor κB ligand (RANKL) by osteoblasts is essential to osteoclastogenesis. Osteoprotegerin (OPG) is a soluble

* Correspondence: helenacanhao@netcabo.pt
† Contributed equally
[1]Rheumatology Research Unit, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Edifício Egas Moniz, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal
Full list of author information is available at the end of the article

receptor for RANKL that prevents RANK/RANKL interaction. Therefore, the RANKL/OPG ratio is critical for the control of bone resorption [10-12]. Increased RANKL/OPG ratio has been described in autoimmune diseases, such as rheumatoid arthritis, and was associated with an increased bone loss [7,13,14]. Taking these arguments into consideration, we have hypothesized that a RANKL/OPG imbalance is also present in SLE patients.

In the present work we aimed to assess the RANKL/OPG balance in SLE patients by quantifying serum OPG and sRANKL levels and their ratio in SLE patients and healthy controls. Additionally, in SLE patients we have looked at predictors of serum levels of these proteins and having as covariates disease features, co-morbidities and medications.

Materials and methods

Patients

Consecutive Portuguese Caucasian SLE women were recruited from the rheumatology outpatient clinics of Hospital de Santa Maria, Lisbon and Hospital Garcia de Orta, Almada, Portugal. All enrolled patients fulfilled the classification criteria of the American College of Rheumatology for SLE (1997) and had normal renal function defined as serum creatinine < 1.5 mg/dl. A control group matched to age, sex and race was also recruited, and was composed of healthy Caucasian female volunteers, who had not been diagnosed with SLE, nor had any inflammatory or bone disease and were not receiving corticosteroids or other medications known to interfere with bone metabolism. In this study, 103 SLE patients and 114 healthy controls were enrolled. This study was approved by the local Ethics Committees and all participants signed a written informed consent.

All participants underwent a standardized clinical and laboratory evaluation [15]. Information about age, height, weight, body mass index (BMI), smoking habits, alcohol intake, menopause, co-morbidities (hypertension, hyperlipidemia, diabetes mellitus, hypo or hyperthyroidism) and medication was collected.

For SLE patients, information considering age at disease diagnosis, disease duration, cumulative clinical manifestations, presence of autoantibodies, current disease activity (evaluated using the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI2K) [16]), and cumulative damage (scored in accordance to the Systemic Lupus International Collaborating Clinics/ACR Damage Index (SLICC) [17]) were also obtained.

Laboratorial determinations

A blood sample was collected from all subjects for measurement of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), lipids (total cholesterol, HDL, LDL and tryglicerides), and anti-dsDNA and anti-Sm antibody titers.

Serum was obtained by blood centrifugation at 1,250 g, 10', at 4°C and then preserved at -80°C until use for sRANKL and OPG quantification.

sRANKL quantification was performed using the ampli sRANKL human ELISA (Immunodiagnostic Systems, Boldon, UK). OPG was quantified using the Bender MedSystems (Vienna, Austria) bead-based assay for quantitative detection of soluble human analytes by flow cytometry. Both protocols were performed according to the manufacturer's instructions.

Statistical analysis

For statistical purposes samples undetectable or below the limit of detection (LOD) were considered to have the lower LOD value supplied by the manufacturer. Results were reported as means (standard deviation), medians (interquartile range) for continuous or proportions for categoric variables. sRANKL and OPG levels and sRANKL/OPG ratio were compared between SLE patients and healthy controls groups using the non-parametric Mann-Whitney test.

Subsequently, the impact of demographic parameters, clinical features, therapeutics and disease characteristics on these proteins and their ratio was investigated for SLE patients using univariate followed by multivariate linear regression analyses. All variables related to the studied outcome in univariate analyses at a P-value < 0.05 were considered potential predictors and entered in multivariate linear regression models. The selection of covariates was stepwise by backward selection, according to the level of significance. Before performing regression analysis, sRANKL, OPG, and sRANKL/OPG ratio were logarithmically transformed for approximation to normality and to approximate the residuals to the normality in multiple linear regressions.

Statistical calculations were performed using Statistical Package for the Social Sciences (SPSS) Statistics Software, v.15.0 (SPSS Inc., Chicago, USA) and a two-tailed P-value < 0.05 was selected as significant.

Results

A total of 103 SLE patients and 114 healthy controls with comparable baseline demographic characteristics and co-morbidities were studied (Table 1). SLE patients had a mean age at disease diagnosis of 35.6 ± 14.4 (range 9.0 to 80.0) years, 8.2 ± 6.6 (range 0.2 to 34.2) years of disease duration, a mean SLEDAI2K of 3.5 ± 4.5 (range 0 to 21) and a SLICC damage score of 0.8 ± 1.4 (range 0 to 8). A total of 60.2% of the patients were currently receiving corticosteroids in a mean daily dose of 12.7 mg.

Table 1 SLE patients and healthy controls characteristics

	SLE patients	Healthy controls	*P*-value
n	103	114	-
Age, years	44.9 ± 14.1	47.4 ± 13.5	0.182
BMI, Kg/m^2	26.6 ± 4.9	26.8 ± 4.9	0.689
Current smokers, n (%)	14 (15.6%)	23 (22.1%)	0.246
Alcohol intake, n (%)	4 (3.9%)	4 (3.6%)	0.602
Postmenopausal, n (%)	44 (49.4%)	57 (53.3%)	0.593
Arterial hypertension, n (%)	52 (50.5%)	47 (42.7%)	0.257
Hyperlipidemia, n (%)	66 (64.1%)	66 (60.6%)	0.596
Diabetes mellitus, n (%)	5 (4.9%)	8 (7.2%)	0.472

Values represent mean ± standard deviation or frequencies of the individuals that presented the characteristic. Differences were assessed using T-test for continuous variables or χ^2 or Fisher's exact test for proportions.

BMI, body mass index; SLE, Systemic Lupus Erythematosus

No significant differences were found between the two groups regarding sRANKL concentration. However, a statistically significant lower value was found for OPG levels in SLE patients ($P < 0.001$) compared to healthy controls. Consequently, an increase in sRANKL/OPG ratio ($P = 0.010$) was found in SLE patients compared to the healthy control group (Table 2).

The adjusted relationship between demographic parameters, clinical features, therapies and disease characteristics with sRANKL, OPG and sRANKL/OPG ratio was further analyzed in SLE patients (Table 3).

In univariate analysis, age, BMI, menopausal status, age at disease onset, the presence of malar rash, and anti-Sm and anti-dsDNA antibody quantifications were significantly associated with sRANKL levels. These possible predictors were included in a multivariate analysis and age (β = -0.232, 95% CI -0.043 to -0.004; $P = 0.017$), malar rash (β = 0.243, 95% CI 0.150 to 1.230; $P = 0.013$), and levels of anti-Sm antibodies (β = 0.227, 95% CI 0.005 to 0.051; $P = 0.018$) were identified as independent predictors of sRANKL levels in SLE patients.

Table 2 sRANKL, OPG and sRANKL/OPG ratio levels in SLE patients and healthy controls

	SLE patients	Healthy controls	*P*-value
sRANKL	0.40 (0.02 to 25.99)	0.40 (0.29 to 67.64)	0.372
OPG	69.02 (17.42 to 500.90)	95.14 (1.56 to 1069.13)	< 0.001
sRANKL/OPG ratio	0.0103 (0.001 to 0.817)	0.0056 (0.000 to 1.866)	0.010

Values represent median (IQR). Differences were assessed by non-parametric Mann-Whitney *U* test.

In 49.5% of SLE patients and 63.2% of healthy controls, the sRANKL levels were below the limit of detection (LOD) of the quantification method. Regarding OPG quantification, there were no samples with concentrations below the LOD.

IQR, interquartile range; OPG, osteoprotegerin; SLE, Systemic Lupus Erythematosus; sRANKL, soluble RANKL

The variables associated with OPG levels in univariate analysis were age, menopausal status, antihypertensive therapy, diabetes mellitus, triglycerides, age at disease onset, arthritis and anti-dsDNA titers. All these potential predictors were included in a multivariate analysis. Diabetes mellitus (β = 0.247, 95% CI 0.117 to 0.984; $P = 0.013$), anti-dsDNA levels (β = -0.239, 95% CI -.004 to 0.000; $P = 0.016$), and triglycerides (β = 0.306, 95% CI 0.001 to 0.004; $P = 0.002$) were found to be independent predictors of OPG levels in the serum of SLE patients.

The same reasoning was applied to the analysis of the sRANKL/OPG ratio in SLE patients. In the univariate analysis age, BMI, menopausal status, lipid-lowering therapy, age at disease onset, the presence of malar rash, pleuritis and the levels of anti-dsDNA and anti-Sm antibodies came out as possible predictors for changes in the ratio values. After multivariate analysis age (β = -0.326, 95% CI -0.055 to -0.015; $P = 0.001$) and levels of anti-Sm antibodies (β = 0.229, 95% CI 0.006 to 0.053; $P = 0.016$) were independently associated with sRANKL/OPG ratio levels in SLE patients.

We found no relationship between sRANKL/OPG ratio and the inflammatory parameters ESR and CRP. In addition, we found no association between the ratio and concomitant medications, such as methotrexate, cyclophosphamide, mycophenolate mofetil or azathioprine. Furthermore, there was also no correlation with corticosteroids (use or actual dose) or disease activity measured by the SLEDAI2K with this ratio.

Although the studied SLE patients presented a wide range of disease duration, this variable did not come out as a predictor of sRANKL, OPG or SRANKL/OPG levels.

Discussion

The present work provides evidence of increased pro-osteoclastogenic stimuli in SLE women as a result of decreased serum OPG levels and increased sRANKL/OPG ratio.

OPG serum levels were lower in SLE patients than in controls and these levels were negatively associated with anti-dsDNA levels, independently from the contribution of multiple confounders. Raised anti-dsDNA levels are associated with active disease, suggesting that patients with active SLE might be more exposed to the effect of RANKL/RANK interaction as a consequence of diminished OPG levels. We have also found a positive association between serum OPG levels and diabetes mellitus, which is in accordance with previous results [18,19]. Gannagé-Yared and colleagues found an inverse correlation between OPG and triglycerides levels, in a nondiabetic, elderly Lebanese male population [20]. Although in a different population, this relation was not

Table 3 Predictors of sRANKL, OPG and sRANKL/OPG ratio levels in SLE patients (after multivariate analysis)

Possible predictors	log(sRANKL)		log(OPG)		log(sRANKL/OPG ratio)	
	Univariate analysis	Multivariate analysis[‡]	Univariate analysis	Multivariate analysis[§]	Univariate analysis	Multivariate analysis*
	β coefficient (95% CI)	β coefficient (95% CI)	β coefficient (95% CI)	β coefficient (95% CI)	β coefficient (95% CI)	β coefficient (95% CI)
	P-value	*P*-value	*P*-value	*P*-value	*P*-value	*P*-value
Age	-0.304 (-0.049 to -0.012)	-0.232 (-0.043 to -0.004)	0.218 (0.001 to 0.015)		-0.363 (-0.058 to -0.019)	-0.326 (-0.065 to -0.015)
	0.002	0.017	0.027		< 0.001	0.001
BMI	-0.249 (-0.126 to -0.016)				-0.211 (-0.123 to -0.005)	
	0.011				0.033	
Menopausal status	-0.210 (-1.131 to -0.006)		0.211 (0.003 to 0.439)		-0.274 (-1.379 to -0.200)	
	0.048		0.047		0.009	
Lipid lowering therapy					-0.214 (-1.451 to -0.068)	
					0.032	
Antihypertensive therapy			0.217 (0.026 to 0.438) 0.028			
Diabetes mellitus			0.271 (0.198 to 1.122)	0.247 (0.117 to 0.984)		
			0.006	0.013		
Triglycerides			0.266 (0.000 to 0.003)	0.306 (0.001 to 0.004)		
			0.011	0.02		
Age at disease onset	-0.268 (-0.045 to -0.008)		0.237 (0.002 to 0.016)		-0.335 (-0.054 to -0.016)	
	0.006		0.016		0.001	
Malar rash	0.251 (0.166 to 1.246)	0.243 (0.150 to 1.230)			0.201 (0.019 to 1.178)	
	0.011	0.013			0.043	
Arthritis			-0.272 (-0.556 to -0.098)			
			0.005			
Pleuritis					-0.197 (-1.555 to -0.010)	
					0.047	
Anti-Sm titers	0.224 (0.003 to 0.051)	0.227 (0.005 to 0.051)			0.264 (0.009 to 0.059)	0.229 (0.006 to 0.053)
	0.026	0.018			0.008	0.016
Anti-dsDNA titers	0.204 (0.000 to 0.007)		-0.208 (-0.003 to 0.000)	-0.239 (-0.004 to 0.000)	0.268 (0.001 to 0.009)	
	0.047		0.043	0.016	0.009	

Multivariate analysis results from multiple linear regression analysis. The total explained variance of the model is (‡) R^2 = 0.185, (§) R^2 = 0.237, and (*) R^2 = 0.175.
OPG, osteoprotegerin; SLE, Systemic Lupus Erythematosus; sRANKL, soluble RANKL

confirmed by our study, since we have found a positive relation between serum OPG and triglycerides.

Serum OPG levels have been scarcely analyzed in the context of SLE. There is a single study reporting higher serum OPG levels in SLE patients, and this relation is even greater in patients with antiphospholipid syndrome, as OPG levels were related to the presence of antiphospholipid antibodies [21]. This relation between OPG and these antibodies was not confirmed by us (data not shown).

On the other hand, urinary OPG levels have been described to be raised in lupus nephritis and correlated with renal disease activity and anti-dsDNA levels [22,23]. However, at this moment it is not clear how

these results can be compared with ours as the relationship between serum and urinary OPG levels is unknown.

We have found sRANKL levels to be similar between SLE and healthy control women, but the sRANKL/OPG ratio was increased in SLE patients as compared to controls at the cost of elevated serum OPG levels in SLE. Interestingly, malar rash and elevated levels of anti-Sm autoantibodies, often present in active disease, were associated with sRANKL serum levels. Moreover, in multivariate analysis levels of anti-Sm antibodies were positively associated with sRANKL/OPG ratio. Studies based on sRANKL are sometimes limited by the high percentage of patients that have undetectable circulating levels, due to the fact that the majority of RANKL is membrane bound. Nevertheless, half of our patients had detectable levels of sRANKL and, importantly, it was possible to determine serum OPG levels in all individuals.

Despite the fact that there are no previous references in the literature to the possible effect of SLE on sRANKL and sRANKL/OPG ratio, an imbalance of this ratio has been described in autoimmune diseases, such as rheumatoid arthritis [13,24]. This finding may be of clinical relevance as the increase of the sRANKL/OPG ratio has been related with increased bone loss in immune mediated inflammatory diseases [12,25]. The independent association of the sRANKL/OPG ratio with anti-Sm autoantibodies and the absence of association with corticosteroid use or dose are particularly relevant, as they are suggestive that SLE *per se* might be important in accelerating osteoclastogenesis and consequently, bone loss.

Conclusions

In summary, we have shown reduced OPG levels and consequently a raised sRANKL/OPG ratio in female SLE patients as compared to healthy controls. An association between anti-dsDNA and OPG levels and between anti-Sm and sRANKL levels and sRANKL/OPG ratio were also observed in SLE patients. Taken together, these observations are suggestive of increased osteoclastic stimuli driven by SLE disease mechanisms.

Abbreviations

Anti-dsDNA: anti-double stranded DNA; Anti-Sm: anti-Smith; BMD: bone mineral density; BMI: body mass index; CRP: C-reactive protein; EDTA: ethylenediamine tetraacetic acid; ELISA: enzyme linked immunosorbent assay; ESR: erythrocyte sedimentation rate; HDL: high-density lipoprotein; LDL: low-density lipoprotein; LOD: limit of detection; OPG: osteoprotegerin; SLE: systemic lupus erythematosus; RANK: receptor activator of nuclear factor κB; RANKL: receptor activator of nuclear factor κB ligand; sRANKL: soluble RANKL; SLEDAI2K: SLE disease activity index; SLICC: systemic lupus international collaborating clinics/ACR damage index; SPSS: statistical package for the social sciences.

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Author details

[1]Rheumatology Research Unit, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Edifício Egas Moniz, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal. [2]Rheumatology Department, Hospital Garcia de Orta, Av. Torrado da Silva, 2801-951 Almada, Portugal. [3]Rheumatology and Bone Metabolic Diseases Department, Hospital de Santa Maria, Av. Prof. Egas Moniz, 1649-035 Lisboa, Portugal.

Authors' contributions

DCF carried out laboratorial protein determinations and participated in the design of the study, statistical analysis and manuscript elaboration. MJS performed clinical evaluation of the patients and participated in the design of study, statistical analysis and manuscript elaboration. IPP helped on laboratorial protein determinations and on manuscript revision. JEF participated in the design of the study and on manuscript revision. HC participated in the design of the study and on manuscript and statistical analysis revision. All authors read and approved the final manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

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IV. Bone disturbances and progression of atherosclerosis in ApoE knockout mice

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Bone disturbances and progression of atherosclerosis in ApoE knockout mice

Diana Carmona-Fernandes[1] , Renata I Casimiro[1], Antti Koskela[2], Mikko Finnilä[3,4], Maria José Santos[1,5], Helena Canhão[6], João E Fonseca[1,7]

[1] JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, Lisbon, Portugal

[2] Cancer Research and Translational Medicine Research Unit, Faculty of Medicine, University of Oulu, Oulu, Finland

[3] Research Unit of Medical Imaging, Physics and Technology, Faculty of Medicine, University of Oulu, Oulu, Finland

[4] Medical Research Center, University of Oulu, Oulu, Finland

[5] Rheumatology Department, Hospital Garcia de Orta, Almada, Portugal

[6] CEDOC, EpiDoc Unit – Epidemiology of Chronic Diseases, Nova Medical School, Universidade Nova de Lisboa, Lisbon, Portugal

[7] Rheumatology and Bone Metabolic Diseases Department, Centro Hospitalar Lisboa Norte, EPE - Hospital de Santa Maria, Lisbon Academic Medical Centre, Lisbon, Portugal

Correspondence should be addressed to Diana Carmona-Fernandes; diana.fernandes@gmail.com

Abstract

Background

Epidemiological evidence supports a link between atherosclerosis and osteoporosis and these conditions might share common pathophysiological mechanisms.

Apolipoprotein E deficient mice ($ApoE^{-/-}$) develop atherosclerotic lesions spontaneously, further aggravated by a high fat diet. Their bone remodeling is also disturbed. We hypothesized that inflammation could be a common contributive factor for vessel and bone disturbances observed in this animal model.

Methods

We evaluated vessels and bones of $ApoE^{-/-}$ and control C57BL/6 (B6) female mice fed an atherogenic diet in five time-points (8, 16, 20, 24 and 28 weeks of age). We quantified the development of atherosclerotic lesions, analyzed gene expression of inflammatory and bone remodeling proteins (IL-1β, IL-6, IL-17A, TNF, RANKL, and OPG), measured serum bone turnover markers (P1NP and CTX-I), performed bone (L3-L4 vertebras) histomorphometric analysis and evaluated biomechanical properties of femurs and tibias by 3-point bending and site specific axial loading of the femoral neck.

Analysis

Statistical evaluation of outcomes between B6 and $ApoE^{-/-}$ groups at each time-point and, within each group, differences between time-points (using parametric t–test or non-parametric Mann Whitney or Kruskal-Wallis tests).

Correlations were investigated using Pearson's or Spearman's correlation coefficient, as appropriate.

Results

Atherosclerotic lesions developed as previously described in ApoE$^{-/-}$ mice, but no significant differences were found in bone histomorphometry or biomechanical properties between ApoE$^{-/-}$ and B6 mice. Also, gene expression (either in bones or aortas) and serum biomarkers were similar in both groups over time.

Additionally, regarding bone histomorphometry analysis, changes observed over time were similar between ApoE$^{-/-}$ and B6 mice. However, the CTX-I/P1NP ratio was significantly increased over time in ApoE$^{-/-}$, but not in B6 mice.

Conclusion

Our study suggests that inflammation is not the principal driver for atherosclerosis progression and bone disturbances in this animal model.

Introduction

Cardiovascular diseases (CV) and osteoporosis (OP) are among the most prevalent health problems and contribute significantly to increased morbidity and mortality of the population.

Atherosclerosis is the major cause of death in Western society and is a result of both genetic and environmental factors [1–3]. Osteoporosis is one of the most common conditions in these populations and is characterized by low bone mass and structural deterioration of bone, leading to an increased fracture risk [4].

Several epidemiological studies have revealed an association between atherosclerosis and osteoporosis. In addition to age, these two diseases share many other common risk factors (such as dyslipidemia, hypertension, type II diabetes, physical activity or menopause) [5, 6], and they may share some common pathophysiological mechanisms [6]. Indeed, systemic inflammation can contribute to the onset of both diseases and thus the mechanisms regulating the effects of inflammation on both vessel and bone could represent potential links between these two conditions.

Several animal models have been used to study the pathogenesis of atherosclerosis. In 1992, Apolipoprotein E deficient mice ($ApoE^{-/-}$) were generated by inactivating the ApoE gene, essential for the transport and metabolism of lipids [7]. These mice spontaneously develop a full range of atherosclerotic lesions distributed throughout the arterial tree, with progression from a foam cell stage to the fibroproliferative stage [1, 8–10]. Female $ApoE^{-/-}$ mice develop atherosclerotic lesions more rapidly [11] and with higher mean lesion area [12] than their male counterparts.

When fed with a Western-type diet, these mice have an accelerated progression of atherogenesis [1, 10]. IL-1β exerts an atherogenic action in this animal model by enhancing the expression of VCAM-1 and MCP-1 in the aorta, which possibly increases the recruitment of monocytes/macrophages to the intima [13, 14]. Additionally, it has been reported that ApoE$^{-/-}$ mice suffer a decrease of bone mass when animals get older [15] and also when they are fed with a high-fat diet [14, 16]. Interestingly, a recent study by Liu and colleagues suggested that this process is associated to changes in inflammatory cytokines [14]. Reduced bone mass has been also observed in C57BL/6 (B6) mice [17, 18] and in other animals [19–21] given a high-fat diet.

There are also some studies that have also reported a high bone mass phenotype in ApoE$^{-/-}$ mice [22, 23]. Nevertheless, Bartelt et al. [22] have shown that apoE deficiency leads to a decreased bone mass and bone formation, while these changes were not present in B6 when fed with high-fat diet.

Based on these findings, we hypothesized that the ApoE$^{-/-}$ mice model is at least partially dependent on the activation of inflammatory pathways and that bone and vascular disturbances in this model share common inflammation related pathways. The goal of this study was to evaluate the progression of bone and vessel disturbances in the ApoE$^{-/-}$ mice model and to test their association with inflammatory markers.

Materials and Methods

Animals

Homozygous ApoE$^{-/-}$ (on a C57BL/6 background) and wild-type (C57BL/6) (B6) mice were obtained from the Charles River Laboratories International (Wilmington, MA, USA). Mice were maintained in a temperature controlled room on a 12-hour light cycle and under specific- pathogen- free (SPF) conditions. This study was guided by the law and ethical regulations related to the use of animals for research (86/609/CEE), reviewed by the IMM Animal Ethics Committee, and licensed by the Direcção-Geral de Alimentação e Veterinária (DGAV).

Experimental design and samples preparation

Only females were included and divided in 5 groups (n=10 for each time-point of sacrifice, at weeks 8, 16, 20, 24 and 28). Starting from 10 weeks old, mice were fed an atherogenic diet (SM R/M High Fat (standard) with 15.2% fat and 12.5 mg/kg cholesterol; Ssniff® Spezialdiäten GmbH, Soest, Germany).

At the end of the experimental periods, mice were deprived of food for 12h. Animals were sacrificed with CO_2 and blood was collected by cardiac puncture, followed by ventricular perfusion with 5ml of PBS. The vessels used for lesion quantification were further perfused with 1ml PFA 3.7%.

The aortas were removed and isolated from the heart up to the iliac bifurcation. The fat tissue was removed and the aortas were either snap-frozen (n=5 for each group) or placed at 4ºC in PFA 3.7% overnight (n=5 for each group).

At the time of sacrifice vertebras (except L3-L4 that were used for histomorphometry studies), femurs and tibias were also harvested and immediately frozen.

Atherosclerotic lesions quantification

After overnight fixation in PFA 3.7%, the aortas were stained with Oil Red O solution at 0.2% (Sigma-Aldrich, St. Lois, MO, USA) and then opened longitudinally, split lengthwise and pinned on a flat surface. Photographs were taken under the stereoscope and reconstructed using Adobe® Photoshop® CC software (version 6.1) and lesions quantification was performed using ImageJ software (version 1.50a).

Bone histomorphometry

The 3rd and 4th lumbar vertebrae (L3-L4) were collected from each animal at sacrifice for histomorphometric analysis, and processed according to the protocol previously described by Vidal and colleagues [24]. Ratio of trabecular bone volume / total tissue volume (BV/TV; %), trabecular thickness (Tb.Th; µm) and trabecular separation (Tb.Sp; µm) were evaluated by standard histomorphometric parameters at 5X magnification.

Bone biomechanical testing

Biomechanical properties of the femurs and tibias were evaluated by three-point bending method using a dual column mechanical testing system (Instron® 3366, Instronis® Corporation, Canton, USA). The load was applied with a speed of 0.155 mm/s and the span length was of 5.5 mm. In addition, biomechanical properties of femoral neck were evaluated in the axial loading in a similar manner by Peng and colleagues [25]. Biomechanical parameters were acquired from the load-deformation curves. Stiffness was defined as the slope of the linear part of the force-deformation curve. Deformation and energy were recorded where bending force reached its highest value. Finally, toughness was evaluated as the area under the curve from contact to the fracture.

RNA isolation from bones and aortas

Bone marrow was flushed out with PBS before femurs were frozen. The aortas and femurs were powdered and the RNA was extracted with TRIzol® reagent (Invitrogen. Life Technologies, Paisley, UK) according to a modified version of the protocol described by Hughes et al. [26]. Briefly, the powder was mixed with TRIzol® and then chloroform was added to solubilize the lipids. A digestion with proteinase K was performed at 55°C, and it was subsequently treated with isopropyl alcohol to precipitate the RNA. The RNA pellet was cleaned with ethanol and then dissolved in RNase/DNase-free water.

The RNA quantification and quality (260nm/280nm and 260nm/230nm ratios) was assessed by absorbance measure using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA).

Quantitative RT-PCR

Total RNA was reverse-transcribed to cDNA according to the manufacturer's instructions (DyNAmo cDNA Synthesis Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA).

The quantitative PCR was performed using DyNAmo Flash SYBR® Green qPCR Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the results measured with the 7500 Fast Real-Time PCR system (Applied Biosystems®, Foster City, CA, USA). The relative expression levels of the target genes (IL-1β, IL-6, IL-17A, TNF, RANKL, and OPG; the sequences of the primers are listed in Table 1) was calculated as a ratio to the housekeeping gene rRNA18S.

Table 1. Housekeeping and target genes primer sequences.

Gene	Forward primer sequence	Reverse primer sequence
IL-1β	5' TGC CAC CTT TTG ACA GTG ATG 3'	5' ATG TGC TGC TGC GAG ATT TG 3'
IL-6	5' GCC TTC TTG GGA CTG ATG CT 3'	5' TGC CAT TGC ACA ACT CTT TTC 3'
IL-17A	5' CCT GGA CTC TCC ACC GCA A 3'	5' TTC CCT CCG CAT TGA CAC AG 3'
TNF	5' AGC CCA CGT CGT AGC AAA C 3'	5' GTG AGG AGC ACG TAG TCG G 3'
RANKL	5' TCC CAT CGG GTT CCC ATA AAG 3'	5' AGG TAC GCT TCC CGA TGT TT 3'
OPG	5' GTG TGG AAT AGA TGT CAC CCT GT 3'	5' CTT GTG AGC TGT GTC TCC GT 3'
rRNA 18s	5' TGT GAT GCC CTT AG 3'	5' CTT ATG ACC CGC AC 3'

IL - Interleukin, TNF - Tumor Necrosis Factor, RANKL - Receptor Activator of Nuclear Factor Kappa-B Ligand, OPG - Osteoprotegerin, rRNA - ribosomal Ribonucleic Acid.

Serum analysis

The levels of P1NP (marker of bone formation) and CTX-I (marker of bone resorption) were determined by Enzyme-Linked Immunosorbent Assay (ELISA) kits (Immunodiagnostic Systems Limited, Tyne & Wear, UK) specific for each one (kit references AC-33F1 and AC-06F1, respectively), performed according to the manufacturer's instructions.

Statistical analysis

Data obtained are shown as means with standard deviations or medians with interquartile range. The normality of distribution was tested by Kolmogorov-Smirnov test.

Differences between groups were determined with parametric t–test or non-parametric Mann Whitney tests (Kruskal-Wallis test when comparing more than two independent groups), according to variables distribution. Correlations were investigated using Pearson's or Spearman's correlation coefficient, as appropriate.

Statistical significance was established for p values ≤ 0.05. All statistical analyses were performed using GraphPad Prism® software, version 6 (GraphPad software, Inc., La Jolla, CA, USA).

Results

Atherosclerotic lesions

The B6 group did not developed atherosclerotic lesions throughout the experimental time period, while the ApoE$^{-/-}$ mice showed signs of atherosclerotic lesions starting at the second time-point of evaluation and progressing until the end-point, being significantly different from the B6 group after week 20 (see Figure 1).

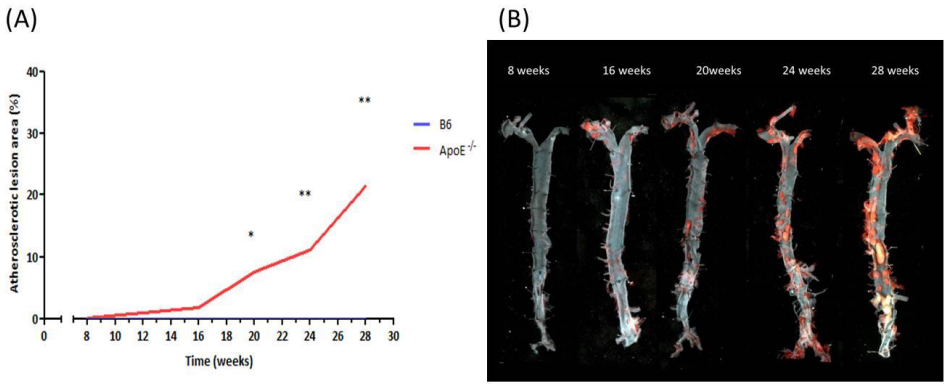
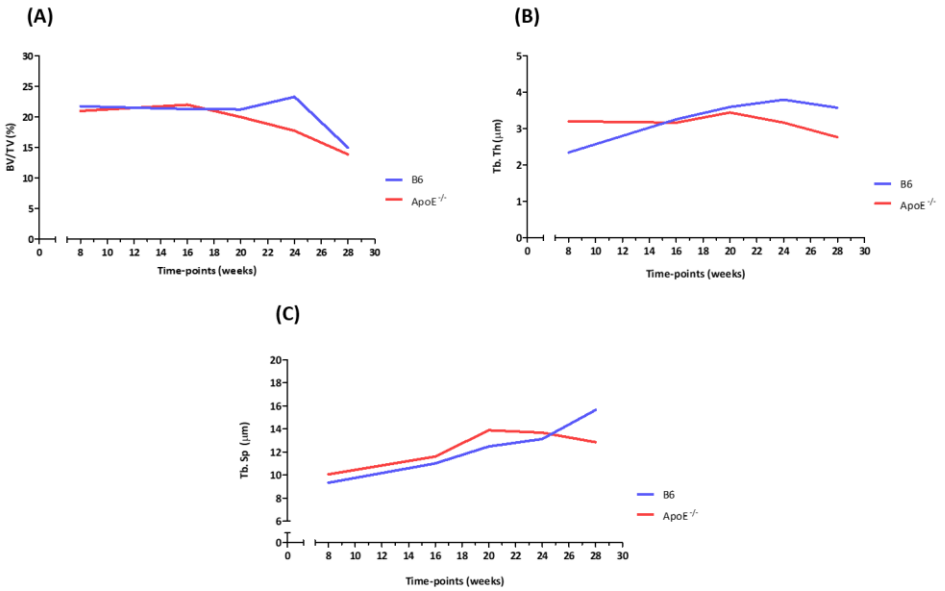


Figure 1. Mice atherosclerotic lesions. (A) Relative areas in B6 and ApoE$^{-/-}$ mice over time; (B) Oil red O staining of ApoE$^{-/-}$ mice aortas. * $p<0.05$; ** $p<0.01$

Bone histomorphometry

Bone histomorphometric parameters (BV/TV, Tb.Th and Th.Sp) had similar patterns of evolution in B6 and ApoE$^{-/-}$ mice, without statistically significant differences between groups (see Figure 2).



222

223 **Figure 2. Bone histomorphometry analysis.** (A) BV/TV progression over time in B6 and
224 ApoE$^{-/-}$ (n=5 for each group at each time-point); (B) Tb.Th progression over time in B6
225 and ApoE$^{-/-}$ (n=5 for each group at each time-point); (C) Th.Sp progression over time in
226 B6 and ApoE$^{-/-}$ (n=5 for each group at each time-point).

227

228 The trabecular thickness (Tb.Th) increased in B6 mice until they were 24 weeks old,
229 although significantly only between 8 and 16 weeks ($p<0.05$) and then slightly decreased
230 up to the last time-point. In the ApoE$^{-/-}$ group the Tb.Th was more stable along time,
231 with a marked decrease between weeks 24 and 28 ($p<0.01$).
232 The trabecular separation (Tb.Sp) increased over time in both groups, with significant
233 differences between the first evaluation (8 weeks old mice) and each time point up to
234 20 weeks of age ($p<0.05$).

235

236

Bone mechanical testing

Bone mechanical behavior progression over time was similar between B6 and ApoE$^{-/-}$ mice (see Figures 3-5 for stiffness and toughness graphs over time). Moreover, only punctual correlations were found between long bone biomechanical properties and lumbar histomorphometry, gene expression or serum remodeling markers (data not shown).

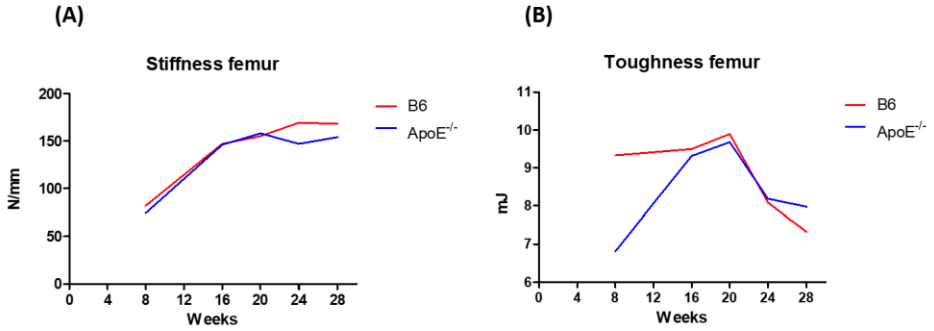


Figure 3. Femurs mechanical testing (three-point bending) results for B6 and ApoE-/- mice over time. n=5 for each group at each time-point.

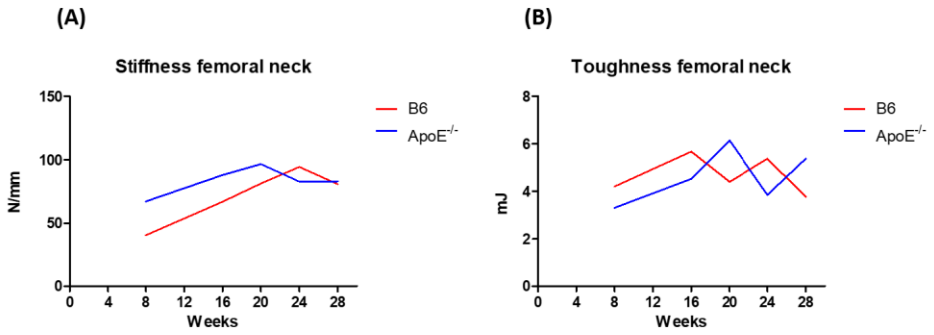


Figure 4. Femoral neck mechanical testing (three-point bending) results for B6 and ApoE-/- mice over time. n=5 for each group at each time-point.

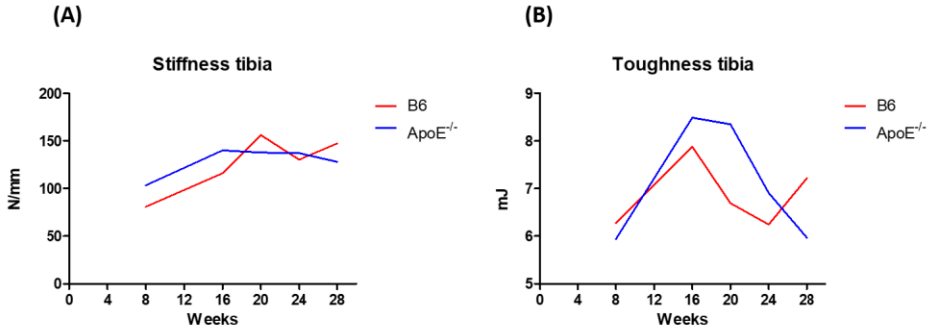


Figure 5. Tibias mechanical testing (three-point bending) results for B6 and ApoE-/- mice over time. n=5 for each group at each time-point.

Quantitative RT-PCR

No significant differences in the gene expression of inflammatory and bone remodeling markers were found between B6 and ApoE$^{-/-}$, either in the aorta or bone, and no

differences were found within groups along time (see Tables S1-S6 for relative gene expression levels).

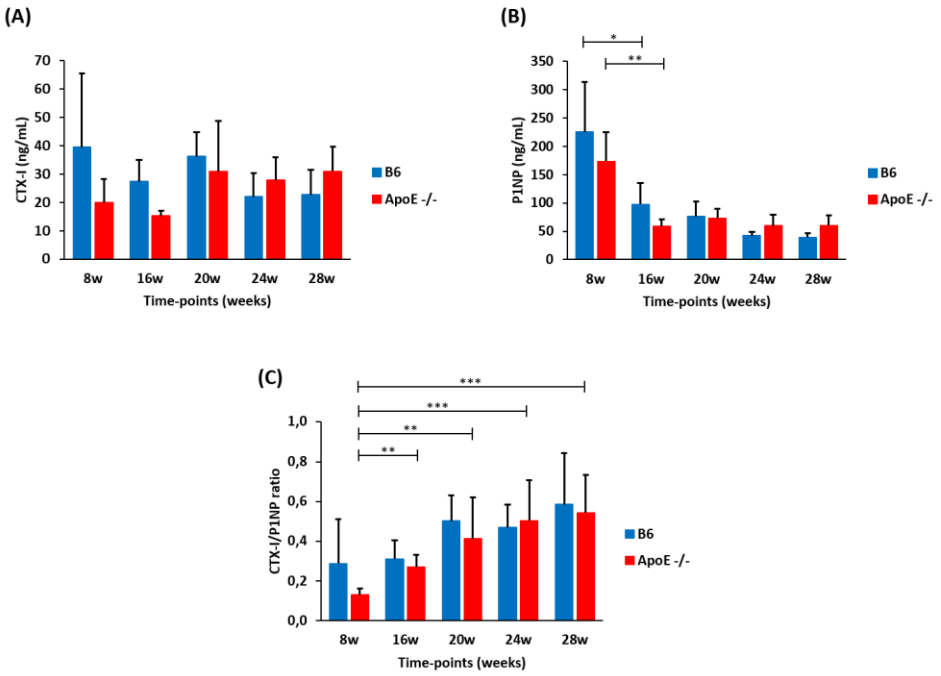
We found no relationship between the studied genes expressed in the two tissues, at any time-point, for either B6 or ApoE$^{-/-}$ mice, neither significant associations between the gene expression in the tissues and the other parameters analyzed (serum levels of bone remodeling biomarkers, histomorphometry and bone mechanical tests parameters). We also searched for correlations ignoring the mice age, but no correlations were found (data not shown).

The expression levels of the tested proteins were significantly higher in the aortas than in bones for all the genes analyzed both in B6 and ApoE$^{-/-}$ mice.

Serum analysis

No statistically significant differences were found for CTX-I, P1NP or CTX-I/P1NP ratio of serum levels between B6 and ApoE$^{-/-}$ mice at any of the time-points analyzed (see Figure 6).

When looking at each strain individually, CTX-I levels (see Figure 6A) had a trend to decrease over time for B6 mice, while for ApoE$^{-/-}$ mice they had a trend to increase with age. P1NP levels (see Figure 6B) decreased with age in both groups, with a significant decrease between weeks 8 and 16 ($p<0.05$ for B6 group, $p<0.01$ for ApoE$^{-/-}$ group) and the CTX-I/P1NP ratio (see Figure 6C) increased after 8 weeks of age in ApoE$^{-/-}$ mice ($p<0.01$ for 8 weeks vs 16 weeks and 20 weeks, and $p<0.001$ for 8 weeks vs 24 weeks and 28 weeks).



286

287 **Figure 6. Serum analysis.** (A) CTX-I levels in B6 and ApoE$^{-/-}$ for each time-point; (B) P1NP

288 levels in B6 and ApoE$^{-/-}$ for each time-point; (C) CTX-I/P1NP ratio in B6 and ApoE$^{-/-}$ for

289 each time-point. * p<0.05; ** p<0.01; *** p<0.001

290

291

Discussion

Our study suggests that inflammation is not the main link between atherosclerosis progression and bone disturbances in the ApoE$^{-/-}$ mouse model.

The number and relative area of the atherosclerotic lesions in ApoE$^{-/-}$ mice aortas increased over time, especially after the introduction of the fat diet. This is in accordance with a previous report by Jawień et al. [1], where an increase in lesion size and accelerated lesion formation was shown due to high fat content diet.

Bone turnover markers in the ApoE$^{-/-}$ group showed an increase in CTX-I/P1NP ratio, as a result from both an increase in CTX-I levels and a decrease in P1NP levels. This is suggestive of an activation of bone turnover throughout time. These data are in accordance with the results obtained for histomorphometric analyses with decreasing bone volume and trabecular thickness, and an increase in trabecular separation over time. This effect on structure, however, was not translated in increased bone fragility as assessed by biomechanical testing. Despite the existence of a trend for lower bone quantity in the ApoE$^{-/-}$ mice as compared to B6 mice, the differences were not statistically significant. It is important to emphasize that the control group was also under a high fat diet, which might have an effect in bone metabolism and explain, at least partly, the lack of significant differences between the ApoE$^{-/-}$ and B6 mice bones. Hirasawa et al. [16] reported that a high fat diet leads to a reduction in bone mineralization. In addition, Parhami et al [5] showed that an atherogenic diet inhibits

315 bone formation by blocking differentiation of osteoblast progenitor cells. Also, the lack
316 of differences between the two groups in the expression of bone remodeling genes
317 (RANKL and OPG) might have been blunted by the diet, in line with the report from Xiao
318 Y et al [27] showing that changes in genes associated with both bone resorption and
319 formation are altered as a consequence of high-fat diet.
320
321 We did not find any differences in the expression of genes related to inflammation (IL-
322 1β, IL-6, IL-17A and TNF) between ApoE$^{-/-}$ and B6 mice. Thus, a dependence of this model
323 on inflammatory pathways previously suggested [14] was not supported by our study,
324 as atherosclerotic lesions developed independently of the expression of genes linked to
325 inflammation. Additionally, neither changes in the expression of these genes were found
326 along time, nor any relationships in the tissue-expression between aorta and bone were
327 observed.
328
329 We have found that the expression levels were significantly higher in the aortas than in
330 bones. Although we have normalized the gene expression levels to the housekeeping
331 gene rRNA 18S, some previous studies have pointed out that housekeeping cannot be
332 assumed to have constant expression across tissue types [28, 29]. In fact, a study
333 regarding the GAPDH mRNA expression (another housekeeping gene widely used) in
334 human tissues has confirmed a marked variability of GAPDH expression between tissue
335 types [30].
336
337

Conclusions

In conclusion, high-fat diet fed ApoE$^{-/-}$ mice developed atherosclerotic lesions since an early stage and they experienced slight disturbances in bone metabolism and structure throughout time, but similar to bone changes observed in B6 mice. However, these effects were not paralleled by significant activation of inflammatory pathways. We suggest that altered lipid transport and high-fat diet, associated with mice aging, may play a more relevant role than inflammation in the development of vessels and bone disturbances in this animal model.

348 # Conflicts of Interest

349

350 The author(s) declare(s) that there is no conflict of interest regarding the publication of

351 this paper.

352

353 # Funding Statement

354

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357 grant from Sociedade Portuguesa de Reumatologia.

358

Supplementary Materials

Tables S1 to S6 provide the relative gene expression levels of inflammatory and bone remodeling markers determined in this work.

Table S1. IL-1β relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment. Data were normalized to 18S rRNA and are represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. IL- Interleukin.

Table S2. IL-6 relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment. Data were normalized to 18S rRNA and are represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. IL- Interleukin.

Table S3. IL-17A relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment. Data were normalized to 18S rRNA and are represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. IL- Interleukin.

Table S4. TNF relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment. Data were normalized to 18S rRNA and are

represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. TNF – Tumor Necrosis Factor.

Table S5. RANKL relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment. Data were normalized to 18S rRNA and are represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. RANKL - Receptor Activator of Nuclear factor Kappa-B Ligand.

Table S6. OPG relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment. Data were normalized to 18S rRNA and are represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. OPG - Osteoprotegerin.

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Table S1. IL-1β relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment.

Groups	Time-points (age in weeks)	IL-1β					
		Aorta			Bone		
		Number of animals (N)	Mean with SD	Median with IQR	Number of animals (N)	Mean with SD	Median with IQR
C57BL/6 (B6)	8	4	9.69±10.65	4.75 [11.93-2.52]	5	0.62±0.33	0.49 [0.93-0.38]
	16	4	3.28±0.97	3.21 83.85-2.64]	5	0.72±0.43	0.72 [0.72-0.44]
	20	4	2.36±1.83	2.36 [3.82-1.35]	4	0.61±0.33	0.54 [0.77-0.38]
	24	4	3.58±0.61	3.60 [4.09-3.10]	5	0.91±0.67	0.81 [1.52-0.22]
	28	5	28.25±25.83	15.36 [55.22-4.74]	5	0.38±0.22	0.46 [0.51-0.21]
ApoE$^{-/-}$	8	5	23.24±29.41	9.68 [27.48-0.34]	5	0.53±0.49	0.20 [0.89-0.15]
	16	4	28.33±21.23	22.74 [35.94-15.13]	5	0.68±0.42	0.56 [0.80-0.40]
	20	4	4.81±5.06	2.64 [6.49-0.96]	5	0.68±0.20	0.66 [0.67-0.62]
	24	4	1.22±0.95	1.10 [1.94-0.38]	5	0.87±0.86	0.22 [1.87-0.16]
	28	4	1.98±1.28	1.27 [2.07-1.19]	5	0.92±0.46	0.77 [0.97-0.63]

Data were normalized to 18S rRNA and are represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. IL- Interleukin.

Table S2. IL-6 relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment.

Groups	Time-points (age in weeks)	IL-6					
		Aorta			Bone		
		Number of animals (N)	Mean with SD	Median with IQR	Number of animals (N)	Mean with SD	Median with IQR
C57BL/6 (B6)	8	3	2.47±1.37	2.95 [3.40-1.78]	5	0.40±0.28	0.33 [0.45-0.18]
	16	5	6.52±3.06	5.95 [9.57-3.89]	5	5.39±5.39	2.69 [10.13-0.48]
	20	5	4.87±1.98	3.96 [5.44-3.92]	5	0.46±0.24	0.52 [0.63-0.33]
	24	4	4.18±1.18	4.50 [4.76-3.91]	5	0.92±0.54	0.92 [0.98-0.25]
	28	5	6.58±4.10	8.23 [10.41-2.82]	5	1.64±1.47	1.39 [1.45-0.11]
ApoE$^{-/-}$	8	5	11.05±10.54	8.08 [14.49-1.46]	4	0.15±0.07	0.15 [0.18-0.12]
	16	4	8.58±4.81	6.93 [10.53-4.99]	5	1.00±1.25	0.24 [1.23-0.10]
	20	5	2.52±1.77	1.71 [2.85-1.58]	5	0.81±0.60	0.55 [0.99-0.53]
	24	5	5.35±3.35	4.19 [7.10-3.05]	4	0.33±0.22	0.33 [0.42-0.24]
	28	5	2.69±1.68	2.14 [3.47-1.51]	5	1.65±1.09	1.11 [1.78-0.98]

Data were normalized to 18S rRNA and are represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. IL- Interleukin.

Table S3. IL-17A relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment.

Groups	Time-points (age in weeks)	IL-17A					
		Aorta			Bone		
		Number of animals (N)	Mean with SD	Median with IQR	Number of animals (N)	Mean with SD	Median with IQR
C57BL/6 (B6)	8	3	3.19±0.21	3.08 [3.28-3.05]	5	0.62±0.32	0.50 [0.65-0.39]
	16	5	2.67±1.44	1.90 [2.72-1.72]	4	1.34±0.43	1.45 [1.68-1.11]
	20	4	1.65±0.87	1.32 [1.82-1.15]	5	1.11±0.57	1.11 [1.22-0.80]
	24	4	2.01±1.50	1.59 [2.91-0.69]	5	1.07±0.87	0.76 [0.99-0.67]
	28	5	4.87±2.42	5.53 [6.593.55]	5	0.77±0.47	0.93 [1.06-0.32]
ApoE$^{-/-}$	8	5	2.96±2.27	2.31 [3.32-1.05]	5	0.56±0.24	0.55 [0.56-0.38]
	16	4	3.39±3.13	2.21 [4.13-1.47]	5	0.42±0.17	0.39 [0.41-0.37]
	20	3	1.95±0.63	1.66 [2.24-1.51]	4	0.55±0.07	0.56 [0.62-0.49]
	24	5	2.78±2.14	2.18 [2.36-1.39]	5	1.70±1.88	0.41 [2.84-0.19]
	28	5	1.97±0.75	2.26 [2.32-1.48]	4	0.64±0.15	0.60 [0.73-0.51]

Data were normalized to 18S rRNA and are represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. IL- Interleukin.

Table S4. TNF relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment.

		TNF					
		Aorta			Bone		
Groups	Time-points (age in weeks)	Number of animals (N)	Mean with SD	Median with IQR	Number of animals (N)	Mean with SD	Median with IQR
C57BL/6 (B6)	8	4	8.33±6.15	8.83 [13.70-3.46]	5	0.30±0.18	0.23 [0.42-0.22]
	16	5	19.84±8.09	19.27 [24.18-14.63]	4	0.43±0.18	0.42 [0.57-0.28]
	20	4	25.26±13.00	23.91 [36.20-12.97]	5	0.16±0.10	0.20 [0.22-0.05]
	24	3	0.76±0.43	0.53 [0.95-0.46]	5	0.38±.35	0.25 [0.37-0.22]
	28	5	18.62±21.13	9.97 [21.73-2.92]	5	0.50±0.38	0.37 [0.88-0.11]
ApoE$^{-/-}$	8	3	0.12±0.08	0.07 [0.15-0.06]	4	0.19±0.07	0.15 [0.20-0.14]
	16	4	8.66±9.26	5.35 [12.04-1.97]	5	0.33±0.25	0.15 [0.55-0.15]
	20	5	3.63±3.99	1.03 [4.97-0.85]	5	0.27±0.15	0.28 [0.29-0.20]
	24	3	2.46±2.45	0.88 [3.40-0.73]	4	0.62±0.41	0.66 [1.01-0.27]
	28	5	10.10±13.84	0.76 [13.38-0.40]	5	0.30±0.19	0.31 [0.46-0.13]

Data were normalized to 18S rRNA and are represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. TNF – Tumor Necrosis Factor.

Table S5. RANKL relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment.

Groups	Time-points (age in weeks)	RANKL					
		Aorta			Bone		
		Number of animals (N)	Mean with SD	Median with IQR	Number of animals (N)	Mean with SD	Median with IQR
C57BL/6 (B6)	8	3	5.25±1.02	4.97 [5.79-4.56]	4	0.28±0.05	0.27 [0.30-0.25]
	16	5	2.85±1.41	2.30 [3.13-2.17]	4	0.94±0.22	0.89 [1.01-0.82]
	20	4	2.49±0.83	2.37 [3.12-1.74]	5	0.60±0.35	0.40 [0.89-0.37]
	24	5	2.74±0.73	3.08 [3.12-1.96]	5	0.51±0.41	0.37 [0.62-0.21]
	28	5	3.31±1.33	2.99 [4.07-2.84]	5	0.82±0.75	0.39 [1.16-0.37]
ApoE$^{-/-}$	8	4	4.36±0.65	4.42 [4.78-4.00]	5	1.00±.87	0.32 [1.91-0.30]
	16	4	5.25±1.25	5.32 [6.18-4.38]	5	0.36±0.10	0.39 [0.44-0.25]
	20	3	1.12±0.07	1.15 [1.17-1.09]	5	0.55±0.39	0.46 [0.51-0.37]
	24	4	1.26±0.69	1.24 1.51-0.99]	4	0.24±0.19	0.15 [0.29-0.10]
	28	5	1.77±1.01	1.56 [1.74-1.08]	4	0.40±0.12	0.40 [0.50-0.30]

Data were normalized to 18S rRNA and are represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. RANKL - Receptor Activator of Nuclear factor Kappa-B Ligand.

Table S6. OPG relative gene expression levels, in aorta and bone, for B6 and ApoE$^{-/-}$ groups at each time-point of experiment.

Groups	Time-points (age in weeks)	OPG					
		Aorta			Bone		
		Number of animals (N)	Mean with SD	Median with IQR	Number of animals (N)	Mean with SD	Median with IQR
C57BL/6 (B6)	8	4	3.07±1.12	3.31 [4.06-2.32]	4	0.37±0.03	0.39 [0.39-0.37]
	16	5	2.28±0.92	2.22 [2.74-1.91]	4	.82±0.27	0.71 [0.88-0.65]
	20	5	2.67±1.30	2.37 [2.78-2.12]	5	0.74±0.45	0.87 [0.91-0.32]
	24	5	2.24±1.25	2.05 [3.42-1.09]	5	0.71±0.59	0.39 [0.33-0.12]
	28	4	2.44±0.86	2.42 [3.28-1.59]	5	0.64±0.42	0.65 [1.01-0.10]
ApoE$^{-/-}$	8	5	1.59±0.59	1.92 [2.00-1.01]	5	0.34±0.06	0.34 [0.39-0.29]
	16	4	2.66±0.43	2.62 [3.03-2.25]	5	0.44±0.15	0.43 [0.52-0.33]
	20	4	1.54±0.76	1.46 [2.18-0.81]	5	0.58±0.18	0.50 [0.74-0.35]
	24	5	1.94±1.00	1.26 [2.86-1.19]	5	0.47±0.31	0.27 [0.62-0.25]
	28	5	2.95±1.51	2.81 [2.91-2.08]	5	1.16±0.28	1.35 [1.36-0.84]

Data were normalized to 18S rRNA and are represented as means ± standard deviation (SD) and medians with IQR (Interquartile range) [75% - 25%]. OPG - Osteoprotegerin.

V. Atherosclerosis and Bone Disease in Humans – results from cadaver donors and endarterectomized patients

Carmona-Fernandes D, Leonardo N, Casimiro RI, Castro A, Barreira S, Santos P, Fernandes AN, Cortes-Figueiredo F, Gonçalves C, Cruz R, Fernandes M, Ivo M, Pedro LM, Canhão H, Fonseca JE, Santos MJ. (Under submission)

Carmona-Fernandes D designed the study and carried out the majority of the experiments, analyzed the data and wrote the manuscript draft.

Atherosclerosis and Bone Loss in Humans – Results from Deceased Donors and Patients Submitted to Carotid Endarterectomy

Carmona-Fernandes D. [a], Leonardo N. [b], Casimiro R.I. [c], Castro A.M. [d], Barreira S.C. [e], Oliveira Santos P. [f], Fernandes A.N. [g], Cortes-Figueiredo F. [h], Gonçalves C.A. [i], Cruz R. [j], Fernandes M.L. [k], Ivo M.[l], Pedro L.M.[m], Canhão H. [n], Fonseca J.E. [o], Santos M.J. [p]

Affiliations:

[a] diana.fernandes@gmail.com; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal.

[b] natachapleonardo@gmail.com; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal.

[c] renata.ip.casimiro@gmail.com; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal.

[d] alicemcastro@gmail.com; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal and Rheumatology and Bone Metabolic Diseases Department, Centro Hospitalar Lisboa Norte, EPE - Hospital de Santa Maria, Lisbon Academic Medical Centre, 1649-035 Lisboa, Portugal.

[e] sofi.barreira@gmail.com; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal and Rheumatology and Bone Metabolic Diseases Department, Centro Hospitalar Lisboa Norte, EPE - Hospital de Santa Maria, Lisbon Academic Medical Centre, 1649-035 Lisboa, Portugal.

[f] santospedro06@gmail.com; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal.

[g] antonio.nfernandes@gmail.com; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal.

[h] filipe.figueiredo@campus.ul.pt; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal.

[i] carolinaamarogoncalves@gmail.com; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal.

[j] rafaelmdacruzmd@gmail.com; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal.

[k] marianafernandeshc@gmail.com; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal.

[l] margarida.ivo@gmail.com; Transplantation Department, Centro Hospitalar Lisboa Norte, EPE - Hospital de Santa Maria, Lisbon Academic Medical Centre, 1649-035 Lisboa, Portugal.

[m] lmendespedro@gmail.com; Vascular Surgery Department, Centro Hospitalar Lisboa Norte, EPE - Hospital de Santa Maria, Lisbon Academic Medical Centre, 1649-035 Lisboa, Portugal.

[n] helenacanhao@gmail.com; CEDOC, EpiDoC Unit, NOVA Medical School, Universidade NOVA de Lisboa, 1150-082 Lisboa, Portugal.

[e] jecfonseca@gmail.com; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal and Rheumatology and Bone Metabolic Diseases Department, Centro Hospitalar Lisboa Norte, EPE - Hospital de Santa Maria, Lisbon Academic Medical Centre, 1649-035 Lisboa, Portugal.

[p] mjps1234@gmail.com; ; JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal and Rheumatology and Rheumatology Department, Hospital Garcia de Orta, 2801-951 Almada, Portugal.

Corresponding author: Diana Carmona-Fernandes, diana.fernandes@gmail.com, JEFonseca Lab – Rheumatology Research Unit, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon Academic Medical Centre, 1649-028 Lisboa, Portugal, Tel: (+351) 217999544, Fax: (+351) 217999459

Figures and Tables: This manuscript includes 4 tables and 1 figure, plus 2 tables as Supplementary Material.

Abstract

Background and aims: Atherosclerosis and osteoporosis frequently occur in the same individual. With this work we aim to understand how changes in bones and arteries correlate with the pathophysiological processes associated with these tissues.

Methods: Gene expression of pro-inflammatory and bone metabolism related proteins were analyzed in arteries and bones from 45 deceased donors. In 139 patients with advanced atherosclerosis submitted to carotid endarterectomy we explored the associations between gene expression in atherosclerotic plaques and bone mineral density (BMD). Additionally, serum levels were measured and plaque morphology and immunochemistry evaluated.

Associations were investigated by the Pearson or Spearman correlation tests, and multivariate regression analyzes were performed when justified.

Results: Gene expression of bone remodeling and inflammatory proteins correlated positively in bone and aorta, independently of age and gender of donors. No association between serum and gene expression levels was found.

The expression of bone formation genes was higher in atheroma plaques from endarterectomized patients with normal BMD comparing with those with low BMD, but we found no differences in expression of inflammatory markers or in serum levels of pro-inflammatory or bone remodeling markers. Immunohistochemical analysis revealed higher CD3 and CD68 scores in patients with normal *vs* low BMD.

Conclusions: We suggest that the relationship between the changes observed in bones and vessels in the context of atherosclerotic disease and osteoporosis, may rely on the intrinsic connection between the tissues involved, independently of the progression of the diseases affecting them.

Keywords: Atherosclerosis; Osteoporosis; Inflammation; Gene expression

Introduction

Atherosclerosis and osteoporosis are among the most prevalent diseases, frequently occurring in the same individual, and their prevalence increases with aging [1],[2].

Atherosclerosis is a chronic inflammatory process that evolves from fatty streaks to the atheroma plaques and causes progressive stenosis of large and medium-sized arteries [3], as a consequence of accumulation of lipids, inflammatory cells, fibrous elements, cellular waste products and calcium[1].

Inflammation, as a key mechanism of atherosclerosis [2], affects the progression of the disease throughout all phases [4]. Endothelial dysfunction and inflammatory lesions are mediated by several pro-inflammatory cytokines. Pro-inflammatory cytokines present in atherosclerotic plaques are produced by monocytes and macrophages [5]. Moreover, high serum levels of IL-1β, IL-6 and TNF [3] are associated with an increase of cardiovascular (CV) risk, as demonstrated by epidemiological studies [6].

Osteoporosis (OP) is a skeletal bone disorder characterized by a decline in bone mineral density (BMD) and microarchitectural deterioration of bone tissue, which causes a reduction in bone strength and, consequently, leads to an increased risk of fracture [7–9]. BMD can be determined by dual x-ray absorptiometry (DXA).

Bone is an active tissue, which is self-remodeled in a coupled action of bone-resorbing cells, osteoclasts, and bone forming cells, osteoblasts [10].

The RANK/RANKL/OPG system, essential to the regulation of bone remodeling [11], is closely related to inflammation. Not only inflammatory cells produce RANKL, but mainly the interaction between RANK and RANKL leads to the release of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF, which increases bone resorption [12].

Despite the central role of the RANK/RANKL/OPG system and the Wnt pathway in bone metabolism, they have also been implicated in the development of atherosclerosis and could be contributing pathways in the regulation of vascular calcification mechanisms [13].

These two diseases share common risk factors [1], as well as molecular and pathophysiological mechanisms [12], although the common underlying mechanisms are

not yet fully understood. Our hypothesis is that there is an association between vessel and bone behavior.

Our aim was to understand how atherosclerotic disturbances observed in arteries are related with alterations in bone. Using samples from deceased donors, we aimed to analyze if a link between bones and vessels exists regarding gene expression patterns of pro-inflammatory cytokines and bone remodeling markers.
Additionally, in a group of patients with advanced atherosclerosis submitted to carotid endarterectomy, we aimed to understand whether gene expression patterns of pro-inflammatory cytokines and bone remodeling markers in atherosclerotic plaques and plaque morphology are related to bone mineral density.

Materials and Methods

Deceased donors' samples

A sample of bone from the iliac crest and a section of the abdominal aorta were collected from 45 deceased donors at the time of organ collection for transplantation, between April 2013 and September 2015. A blood sample was also obtained. From a subgroup of 7 patients (4 men and 3 women), an additional sample of adipose tissue was collected. Due to confidentiality aspects, no clinical information beyond age and gender could be retrieved, but all of them had clearance to be organ donors, which means that they did not present major health issues at the time of death.

Endarterectomized Patients/Advanced Atherosclerosis Samples

Atherosclerotic plaques and fasting blood samples were collected from 139 patients submitted to carotid endarterectomy surgery between May 2012 and May 2015. All patients performed a dual X-ray absorptiometry (DXA) and were classified with osteoporosis, osteopenia or normal BMD according to the WHO classification criteria [14].

A structured protocol was applied to all patients for recording demographic data, CV risk factors, history of previous factures, personal and family history of OP, other comorbidities, lifestyle and nutritional habits, and past and current medication.

The 10-year probability of major osteoporotic fracture (FRAX 1) or hip fracture (FRAX 2) were calculated using the FRAX® tool validated for the Portuguese population [15] and the 10-year probability of CV disease death (SCORE) was calculated using the HeartScore® tool adapted for low risk countries [16].

Body mass index (BMI) values were used to classify patients as overweight (BMI ≥ 25 Kg/m^2) or obese (BMI ≥ 30 Kg/m^2), according to WHO classification criteria [17].

Calcium intake was calculated according to Cosman F. et al. [18], based on a food frequency questionnaire by calculating consumption of milk, yogurt and cheese in mg/day.

This study was approved by the Ethics Committee of Hospital de Santa Maria and patients signed written informed consent prior to any protocol-specific procedure. All

proceedings were conducted in accordance with the regulations governing clinical trials such as the Declaration of Helsinki, as amended in Fortaleza, Brazil (2013) [19].

Biologic samples collection and storage

Endarterectomy samples - the central and visually more developed plaque was sectioned crosswise over the longitudinal axis in two sections: one for RNA extraction was fragmented in smaller pieces and immediately frozen in liquid nitrogen (snap-frozen) and stored at -80ºC and the other for histology and immunohistochemistry was frozen in OCT (optimal cutting temperature compound) and stored at -80ºC.

Deceased organ donor samples – Bone biopsies and aorta sections were processed the same way as the atheroma plaques.

Blood samples - were centrifuged upon arrival to the lab and the serum was collected and stored at -80ºC for later analysis.

RNA isolation from bones and aortas

Samples were reduced to a fine powder with a pestle and mortar cold with liquid nitrogen and the RNA was extracted with TRIzol® (Invitrogen™), according to a modified version of Hughes et al. protocol [20]. Briefly, the powder was placed in TRIzol® and homogenized, and chloroform was added to solubilize the lipids. A digestion with proteinase K was performed at 55ºC, and it was subsequently treated with isopropyl alcohol to precipitate the RNA. The RNA pellet was cleaned with ethanol and then dissolved in RNase/DNase-free water.

The RNA quantification and quality was obtained by absorbance measured using the NanoDrop® ND- 1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA).

Quantitative RT-PCR

Total RNA was reverse-transcribed to cDNA according to the manufacturer's instructions (DyNAmo cDNA Synthesis Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA).

The quantitative PCR was performed using DyNAmo Flash SYBR® Green qPCR Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the results measured with Rotor

Gene 6000 (Qiagen, Germany) for deceased donors' samples and with 7500 Fast Real-Time PCR System (Applied Biosystems®, Foster City, CA, USA) for advanced atherosclerosis patients' samples. The sequences of the primers used (*IL-1β, IL-6, IL-17A, TNF, RANKL, OPG, COL1, CTSK, OCL, TRAP, CBFA1, DKK1, SOST, ADIPOQ* and *ADIPOR1*) are listed in Tables S1 and S2 (Supplementary Material). The efficiency of qPCR was analyzed using the standard curve method, as described previously [21]. The values obtained were normalized with the housekeeping gene 18S rRNA.

Cytokine and bone markers quantification

RANKL and OPG serum levels were determined using Biomedica ELISA (Enzyme-Linked Immunosorbent Assay) (Cat. No. BI-20462 and BI-20403, respectively), and CTX (C-terminal telopeptide of type 1 collagen) and P1NP (procollagen type 1 N propeptide) with SunRed Biological Technology (Cat. No. 201-12-1350 and 201-12-2130, respectively). Data were acquired in the microplate reader Infinite® M200 (Tecan).

CBA (Cytometric Bead Array) determination was performed for pro-inflammatory cytokines, IL-1β, IL-6, IL-17A and TNF (BD™ CBA Enhanced Sensitivity Flex Set; Cat. No. 561509 (for IL-1β), Cat. No. 561512 (for IL-6), Cat. No. 562143 (for IL-17A), and Cat. No. 561516 (for TNF)) and data were collected in the Accuri™ C6 flow cytometer from BD™ Biosciences.

Histological evaluation

Frozen plaques were sectioned crosswise over their longitudinal axis using a cryostat, and the major segment was used for histological analysis.

Alizarin Red S (Sigma, Missouri, USA) histological staining was used for calcium determination. The protocol was adapted (no de-paraffinization needed, slides were slowly immersed in distilled water) from IHC World website [22].

Immunohistochemical staining of the plaques was also performed with CD3 (eBioscience, San Diego, USA), CD68 (eBioscience, San Diego, USA) and Adiponectin (Boster Biological Technology, Pleasanton, USA) antibodies. Tissue sections were incubated with the primary antibody and with EnVision+ (Dako, Glostrup, Denmark). Color was developed in solution containing diaminobenzidine-tetrahydrochloride

(Sigma, Missouri, USA), 0.5% H_2O_2 in phosphate-buffered saline buffer (pH 7.6). Slides were counterstained with hematoxylin and mounted [23].

Histological and immunohistochemical evaluations were performed using a semi-quantitative score of 0 to 3 (0 – 0 to 10% staining; 1 – 10 to 50% staining; 2 - 50 to 75% staining; 3 - more than 75% staining). Slides were observed in a ZEISS Primo Star (ZEISS, Oberkochen, Germany) microscope.

Statistical analysis

Statistical analysis was performed using IBM SPSS version 20. Quantitative variables are described as means and standard deviation and qualitative variables as percentages and absolute frequencies.

Statistical significance was considered for a two-tailed $p<0.05$ and the confidence interval for all statistical analysis was 95%. The normality of the distribution for continuous variables was evaluated using Kolmogorov-Smirnov test. For non-normally distributed variables, non-parametric tests (Spearman's test) were used while parametric tests (Pearson's test) were applied for variables with normal distribution. Comparisons between groups were performed using Student's t test, Mann-Whitney or Kruskal-Wallis test, as appropriate.

When justifiable, multivariable linear regression analyses with backward selection of covariates was performed.

Data availability

The data that support the findings of this study are available in Zenodo repository with the identifier 10.5281/zenodo.1403777.

Results

Deceased donors

Population description

A total of 45 donors were included in this study with ages ranging between 15 and 80 years old: 23 men with 49.6±17.8 years old and 22 women with 61.1±12.9 years old. The aortas were evaluated macroscopically and in 6 of them (about 13%) were visible calcifications.

Quantitative RT-PCR

A positive correlation between gene expression levels in bone and aorta samples was observed for almost all studied genes, except *OCL* gene (p>0.05) (see figure 1). The correlations found were classified, as described by Evans [24], as weak for the *TNF* gene; moderate for the *IL-1β, IL-6, RANKL, COL1A1, CTSK, TRAP, ADIPOQ* and *ADIPOR1* genes; and strong for the IL-*17A, OPG, CBFA1, DKK1* and *SOST* genes.

Adipose tissue samples were used to verify if the association found between bone and aorta samples was present in other tissues or if this was specific to the two tissues of interest. When we correlated the gene expression levels of bone and aorta samples individually with adipose tissues samples we found no association.

No significant differences in the gene expression of inflammatory and bone remodeling markers in aorta, bone or adipose tissue samples were found between men and women neither in association with age.

Figure 1. Correlations of gene expression levels between bone and aorta samples.

IL – Interleukin; TNF – Tumor necrosis factor; RANKL - Receptor Activator of NF-kB Ligand; OPG – Osteoprotegerin; COL1A1 – Collagen type I; CTSK – Cathepsin K; OCL – Osteocalcin; TRAP – Tartrate resistant acid phosphatase; CBFA1 - Core-Binding Factor Alpha I; DKK1 - Dickkopf-related protein 1; SOST - Sclerostin; AdipoQ - Adiponectin; AdipoR1 - Adiponectin receptor 1.

Serum cytokines and bone markers analysis

No significant correlations were found between serum levels and gene expression levels, either from bone, aorta or adipose tissue samples, for any of the proteins studied.

We found that IL-1β levels were significantly higher in men than in women (442.7 fg/mL [range 179.3-4569] vs 89.4 fg/mL [range 38.9 – 504.9]; p=0.011), independently of donors' age. No differences could be depicted for any proteins in relation to donors' age.

Atherosclerosis patients

Population description

A total of 139 patients with advanced atherosclerosis were included in the study, where 95 (68.3%) were men, 70.3±8.7 years old, and 44 (31.7%) were women, 71.5±9.6 years old.

We have further compared the clinical characteristics, co-morbidities and therapies between patients with normal BMD (t-score >-1) and patients with low BMD (t-score ≤ -1; includes osteopenia and osteoporosis diagnosis). The details are listed in Table 1. Patients with lower BMD were older (p=0.001), had lower BMI levels (p<0.001) and had a higher probability of fracture evaluated by FRAX (p<0.001). They did not differ significantly in any of the other variables evaluated.

Table 1 – Clinical characteristics, co-morbidities and therapies of atherosclerosis patients with normal and low BMD.

Quantitative RT-PCR

Regarding bone remodeling markers in the atheroma plaques, we found that genes associated with bone formation were expressed at higher levels in patients with normal BMD than in patients with low BMD (Table 2). Specifically, *CBFA1* (0.98±0.08 vs 0.71±0.06, p=0.009) and *OCL* (1.27±0.12 vs 0.83±0.08, p=0.003), and this difference was significant independently of age and gender.

Table 2 – Gene expression levels in the atheroma plaques of atherosclerosis patients with normal and low BMD.

The gene expression of inflammatory markers (*IL-1β, IL-6, IL-17A* and *TNF*) in the plaques did not differ or relate to any of the patient's characteristics (demographic characteristics, lifestyle habits, co-morbidities or medication).

Additionally, we found that *OCL* gene expression levels in the plaques is higher in patients with dyslipidemia (1.12±0.73 vs 0.74±0.69, p=0.007) and in patients under statins therapy (1.11±0.74 vs 0.88±0.81, p=0.044). However, in multivariate analysis *OCL* gene expression levels remained significantly associated only with BMD.

Serum cytokines and bone markers analysis

Regarding serum levels analyzed, we did not find differences between patients with normal or low BMD (Table 3).

No other relations were found between measured serum levels and patients' characteristics, neither with gene expression levels.

Table 3 – Serum levels of atherosclerosis patients with normal and low BMD.

Histological evaluation

Plaque CD3 and CD68 immunohistochemistry scores were higher in patients with normal BMD than in patients with low BMD (Table 4). Additionally, CD3 (0.82±0.97 vs 0.34±0.53, p=0.006) and CD68 (0.89±0.89 vs 0.45±0.63, p=0.005) immunohistochemistry scores were higher in male patients. In the independent analysis, CD3 (ρ=-0.221, p=0.009) and CD68 (ρ=--0.181, p=0.033) immunohistochemistry scores were inversely related to patients age.

In a multivariate analysis, CD3 values are dependent on age and gender (β=-0.228, p=0.006 and β=-0.255, p=0.002, respectively), but not BMD; on the other hand, CD68 values are dependent on gender and BMD (β=-0.230, p=0.007 and β=-0.203, p=0.016,

respectively), but not age. No other associations with clinical or demographic data were found for any of the histological studies.

Table 4 – Histological scores of atherosclerosis patients with normal and low BMD.

No significant correlations were found between any of the histological studies performed (alizarin red S, CD3, CD68 or adiponectin) and the results of the ELISAs or gene expression quantifications.

Discussion

With our work we aimed to understand the role of inflammation in the relationship between bone changes observed in osteoporosis and vascular pathology, namely atherosclerosis.

Regarding several genes related to inflammation and bone remodeling we found that, at the gene expression level, there is a positive correlation between bones and vessels, specifically the aorta, suggesting a link between these two systems. In addition, the observed correlation did not extend to adipose tissue, which supports that this is not a widespread finding.

An increase in atherosclerotic lesions' calcification has been associated with bone loss [25], as described in $OPG^{-/-}$ mice, a model of osteoporosis [26], where two thirds of these mice developed aortic calcifications concomitantly with early onset OP [27]. Considering that, we could expect an inverse relation in the expression of bone remodeling proteins between bones and vessels, but instead we observed a direct relationship. Can there be a parallel expression in normality that is reversed in pathology? Some clues in this direction have already been reported by Schweighofer et al. [28] that have described a change in the gene expression of calcification regulators with atherosclerosis progression.

Of interest, vessels were from deceased donors with unknown CV pathology, and although some had atheroma plaques in the aorta (data not shown), these plaques are not present in the majority, increasing the interest and relevance of having bone remodeling proteins expressed on these tissues.

No differences on the gene expression pattern with age or gender were found in deceased donors' samples, suggesting that the relations described above do not vary significantly throughout life neither between women and men, pointing that, in pathologic processes, bone and vessel disturbances are possibly linked due to an intrinsic connection between the tissues involved. To the best of our knowledge there

are no previous reports of any relation between the expression of these genes, either in the vessels or in bones, with age or gender.

Another point that reinforces this relation between tissues is the fact that the gene expression levels are not related to the circulating levels of the same proteins. In fact, regarding ELISAs results, we only found that IL-1β levels were higher in men (independently of age) than in women, which is in accordance with previous results reported by other groups [29,30]. Previous studies regarding serum determination of some cytokines reported changes with gender and ethnicity [31], and also with sampling time [32]. The variability of cytokines is also due to genetic, non-heritable (including age) and microbial factors [33].

In the second part of our work we have used atherosclerotic plaques from patients with advanced atherosclerosis that were submitted to endarterectomy surgery and where bone status evaluation was performed by DXA. We found that more than 45% of the enrolled patients had low BMD, which is in accordance with the age range of this population and with previous studies where bones and vessels were both evaluated by imaging methods[34,35].

We found that the expression of bone formation genes (*CBFA1* and *OCL*) on atherosclerotic plaques is lower in those patients who have decreased BMD. Could this be an indication that in these patients the plaques have a lower tendency to calcify? Our results do not corroborate this hypothesis, as we have not found any differences in the calcium content, determined by Alizarin Red S staining, between patients with normal or low BMD.

Although the presence of these proteins in plaques is in accordance with previous studies showing that calcified plaques composition share some features of bone structure [36], these results seem contradictory to previous studies [37–39] that reported an association between low BMD and vascular calcification. These differences might be related to different stages of plaque development between those studies and ours, as calcification can begin at any point of plaque formation and progression [36].

Statins are widely used to reduce low-density lipoproteins (LDL) levels and inhibit the inflammatory processes directly [40], and have been suggested as potential agents in the management of osteoporosis, namely through an antiresorptive effect on bone cells [41]. Although 74.1% of the patients included in our study are under statins therapy, we found that a high percentage (about 46%) have decreased BMD. However, we found that gene expression levels of osteocalcin (expressed by differentiated osteoblasts, the bone formation cells) on the atherosclerotic plaques are higher in patients with dyslipidemia and in those under statins therapy. Previous studies have pointed that statins therapy influence plaques' composition, in addition to their effects on plaque regression [42,43]. In fact, Mujaj and colleagues have reported that statins were associated with a higher presence of calcification in carotid plaques [44]. Our data seems to point to a dual effect of statins therapy through the presence and activity of osteoblasts, positively affecting bone metabolism while simultaneously increasing plaques' calcification.

When analyzing the results of immunohistochemical staining performed on the atherosclerotic plaques of our patients, CD3 scores were higher in patients with normal BMD and in male patients, and CD68 scores were higher in males and in younger patients. Both CD3 (expressed in T-cells) and CD68 (mainly expressed in macrophages) are inflammation related, suggesting that atheroma plaques from men have higher levels of inflammation, as previously proposed [45,46]. Low BMD was associated with echogenic (more calcified) plaques [47,48], and calcification and inflammation are thought to be active at different stages of disease progression [49], with inflammation at early phases and calcification predominantly later [46,50]. Thus, patients with low BMD are expected to have lower inflammation in the plaques, which is in accordance with our immunohistochemical results regarding CD3.

This work is a cross-sectional evaluation of both deceased donors and atherosclerosis patients, which confers some limitations to our study, such as the impossibility to evaluate causality mechanisms. Despite the limitation resulting from the lack of clinical information of deceased donors, these samples allowed us to directly evaluate gene expression on bone and aortas from the same subjects and not only through indirect

diagnostic methods. Regarding atherosclerosis patients, it was not possible to have access to bone tissue of these patients to perform the study directly on the tissue affected. However, contrary to most studies, we had access to the carotid atheroma plaques and not only to ultrasound evaluation of atherosclerosis, and all the patients had their BMD evaluated by DXA.

Conclusions

We have described a positive correlation between bones and aortas concerning the expression of inflammation and bone remodeling genes. It is in fact interesting to verify that bone-remodeling genes are not only expressed on atherosclerotic plaques, but also on vessel walls, regardless of the presence of plaques.

Additionally, the atheroma plaques of patients with low BMD present lower levels of bone formation markers (*CBFA1* and *OCL*) and a lower score of CD3 and CD68 immunostainings than those with normal BMD.

With this study, we suggest that the relationship between the changes observed in bones and vessels in the context of atherosclerotic disease and osteoporosis, may rely on the intrinsic connection between the tissues involved, independently of the progression of both diseases.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Authors Contributions

DCF was involved in the study design and performed the laboratory work, data analysis, and manuscript writing; NL, RIC, ANF, FCF and RC were involved in the laboratory work and critical revision of the article; AMC, SCB, POS, ANF, FCF, CAG, RC and MLF in data acquisition and critical revision of the article; MI and LMP were involved in samples collection and critical revision of the article; HC, JEF and MJS were involved in the study design, data analysis and critical revision of the article. All authors read and approved the final manuscript.

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Supplementary Material

Table S1 - Housekeeping and target genes primer sequences used for deceased donors' samples

Table S2 - Housekeeping and target genes primer sequences used for endarterectomy patients' samples

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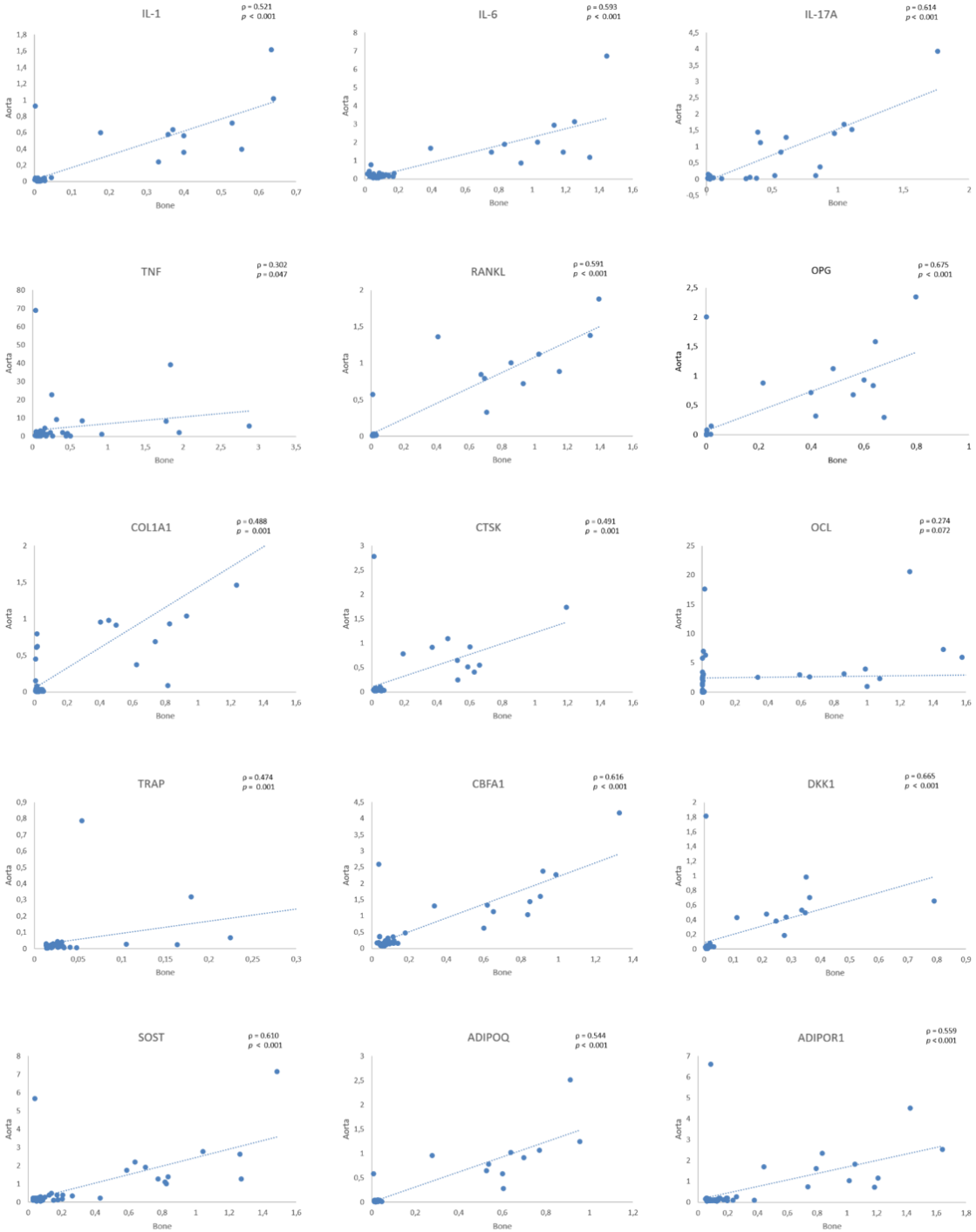
IL-1
ρ = 0.521
p < 0.001
IL-6
ρ = 0.593
p < 0.001
IL-17A
ρ = 0.614
p < 0.001
TNF
ρ = 0.302
p = 0.047
RANKL
ρ = 0.591
p < 0.001
OPG
ρ = 0.675
p < 0.001
COL1A1
ρ = 0.488
p = 0.001
CTSK
ρ = 0.491
p = 0.001
OCL
ρ = 0.274
p = 0.072
TRAP
ρ = 0.474
p = 0.001
CBFA1
ρ = 0.616
p < 0.001
DKK1
ρ = 0.665
p < 0.001
SOST
ρ = 0.610
p < 0.001
ADIPOQ
ρ = 0.544
p < 0.001
ADIPOR1
ρ = 0.559
p < 0.001
Aorta
Bone

Table 1 – Clinical characteristics, co-morbidities and therapies of atherosclerosis patients with normal and low BMD

Characteristic	Normal BMD (N=69)	Low BMD (N=64)	p-value
Age (years)	67.9±8.6	73.0±8.6	0.001
Gender	49 M / 20 F	41 M / 23 F	0.392
Hypertension	59 (85.5%)	49 (76.6%)	0.187
Previous CV event	44 (63.8%)	37 (57.8%)	0.482
Alcohol (above 3U/day)	19 (27.5%)	15 (23.4%)	0.554
Calcium intake (mg/day)	848.6±498.27	896.8±468.9	0.372
Actual / Previous smokers	9 (13.0%) / 28 (40.6%)	8 (12.5%) / 29 (45.3%)	0.850
Active lifestyle	7 (10.1%)	5 (7.8%)	0.724
BMI (Kg/m^2)	28.5±4.4	25.5±4.0	< 0.001
Overweight / Obese	31 (44.9%) / 23 (33.3%)	24 (37.5%) / 9 (14.1%)	0.005
Previous fragility fracture	6 (8.7%)	6 (9.4%)	0.891
Parental fragility fractures	3 (4.3%)	8 (12.5%)	0.095
Dyslipidemia	59 (85.5%)	53 (82.8%)	0.833
Type II Diabetes mellitus	20 (28.9%)	17 (26.6%)	0.716
Rheumatoid arthritis	0 (0%)	4 (6.3%)	0.051
FRAX 1 (%)	3.8±2.4	9.0±1.0	< 0.001
FRAX 2 (%)	1.0±1.1	4.6±6.8	< 0.001
SCORE (%)	3.2±1.6	2.8±1.3	0.168
Corticotherapy (> 3 months)	4 (5.8%)	8 (12.5%)	0.167
Statins therapy	52 (75.4%)	46 (71.9%)	0.651

Quantitative variables are presented as means ± SD and qualitative variables as absolute values and proportions of total (%). BMD – bone mineral density; CV – cardiovascular; BMI – body mass index; FRAX 1 - 10-year probability of a major osteoporotic fracture; FRAX 2 - 10-year probability of hip fracture; SCORE - 10-year probability of CVD death.

Table 2 – Gene expression levels in the atheroma plaques of atherosclerosis patients with normal and low BMD

Gene	Normal BMD (N=69)	Low BMD (N=64)	p-value
IL-1β	1.86±1.57	1.59±1.18	0.617
IL-6	0.65±0.35	0.60±0.31	0.527
IL-17A	1.62±1.56	1.93±1.95	0.360
TNF	137.2±172.2	112.2±175.6	0.262
RANKL	1.46±0.95	1.23±0.91	0.137
OPG	2.66±1.99	2.29±1.82	0.288
RANKL/OPG	0.46±0.26	0.44±0.28	0.767
COL1A1	0.89±0.54	0.72±0.48	0.065
CTSK	0.81±0.49	0.77±0.50	0.477
OCL	1.27±0.84	0.84±0.54	0.009
TRAP	6.93±10.51	5.69±7.54	0.442
CBFA1	0.98±0.63	0.71±0.47	0.015
DKK1	3.63±2.67	3.04±2.66	0.116
SOST	2.89±1.89	2.89±2.17	0.647
AdipoQ	34.4±47.1	27.2±35.6	0.671
AdipoR1	1.91±1.16	1.62±1.01	0.223

Variables are presented as means ± SD. IL – Interleukin; RANKL - Receptor Activator of NF-kB Ligand; OPG – Osteoprotegerin; COL1A1 – Collagen type I; CTSK – Cathepsin K; OCL – Osteocalcin; TRAP – Acid phosphatase tartrate resistant; CBFA1 - Core-Binding Factor Alpha I; DKK1 - Dickkopf-related protein 1; SOST - Sclerostin; AdipoQ - Adiponectin; AdipoR1 - Adiponectin receptor 1.

Table 3 – Serum levels of atherosclerosis patients with normal and low BMD

Protein	Normal BMD (N=69)	Low BMD (N=64)	p-value
IL-1β (fg/mL)	14.8±49.1	12.9±35.8	0.780
IL-6 (fg/mL)	2139.5±1730.7	1982.3±1953.7	0.235
IL-17A (fg/mL)	17.9±50.2	15.3±30.8	0.380
TNF (fg/mL)	11.8±47.7	8.02±37.2	0.352
RANKL (pmol/L)	0.022±0.019	0.019±0.015	0.389
OPG (pmol/L)	8.11±5.45	7.97±5.44	0.885
RANKL/OPG	0.0033±0.0040	0.0034±0.0040	0.990
CTX (ng/mL)	30.8±6.9	30.8±8.3	0.865
P1NP (ng/mL)	130.5±75.2	135.6±75.4	0.678
CTX/P1NP	0.21±0.09	0.23±0.08	0.288
Adiponectin (ng/mL)	114909.4±93591.1	130745.4±99158.3	0.403

Variables are presented as mens ± SD. IL – Interleukin; RANKL - Receptor Activator of NF-kB Ligand; OPG – Osteoprotegerin; CTX – C-terminal telopeptide of type 1 collagen; P1NP – procollagen type 1 N propeptide.

Table 4 – Histological scores of atherosclerosis patients with normal and low BMD

Molecule	Normal BMD (N=69)	Low BMD (N=64)	p-value
Alizarin Red S	1.94±0.95	1.92±1.04	0.991
CD3	0.78±0.91	0.47±0.76	0.020
CD68	0.91±0.84	0.55±0.79	0.004
Adiponectin	1.16±0.90	1.13±0.90	0.780

Variables are presented as mens ± SD. CD – Cluster of differentiation.

Table S1 - Housekeeping and target genes primer sequences used for deceased donors' samples

Gene	Forward primer sequence	Reverse primer sequence
18S rRNA	GGAGTATGGTTGCAAAGCTGA	ATCTGTCAATCCTGTCCGTGT
IL-1β	TACCTGTCCTGCGTGTTGAA	TCTTTGGGTAATTTTTGGGATCT
IL-6	GATGAGTACAAAAGTCCTGATCCA	CTGCAGCCACTGGTTCTGT
IL-17A	TTCCCCCGGACTGTGATGGTCA	CAGGGTCCTCATTGCGGTGGAGA
TNF	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA
RANKL	AGAGAAAGCGATGGTGGATG	TATGGGAACCAGATGGGATG
OPG	CGCTCGTGTTTCTGGACAT	GTAGTGGTCAGGGCAAGGG
COL1A1	ACGAAGACATCCCACCAATC	AGATCACGTCATCGCACAAC
CTSK	GCCAGACAACAGATTTCCATC	CAGAGCAAAGCTCACCACAG
OCL	CCAGGCAGGTGCGAAG	TCAGCCAACTCGTCACAGTC
TRAP	CGGCCACGATCACAATCT	GCTTTGAGGGGTCCATGA
CBFA1	CGGAATGCCTCTGCTGTTA	TCTGTCTGTGCCTTCTGGGT
DKK1	CAGGCGTGCAAATCTGTCT	AATGATTTTGATCAGAAGACACACATA
SOST	AGACCAAAGACGTGTCCGAG	GGGATGCAGAGGAAGTC
AdipoQ	GGTGAGAAGGGTGAGAAAGA	TTTCACCGATGTCTCCCTTAG
AdipoR1	TTGTGTACAAGGTCTGGGAGG	GATGCTCTTGAAGCAAGCCC

IL – Interleukin; TNF – Tumor necrosis factor; RANKL - Receptor Activator of NF-kB Ligand; OPG – Osteoprotegerin; COL1A1 – Collagen type I; CTSK – Cathepsin K; OCL – Osteocalcin; TRAP – Tartrate resistant acid phosphatase; CBFA1 - Core-Binding Factor Alpha I; DKK1 - Dickkopf-related protein 1; SOST - Sclerostin; AdipoQ - Adiponectin; AdipoR1 - Adiponectin receptor 1.

Table S2 - Housekeeping and target genes primer sequences used for endarterectomy patients' samples

Gene	Forward primer sequence	Reverse primer sequence
18S rRNA	GGAGTATGGTTGCAAAGCTGA	ATCTGTCAATCCTGTCCGTGT
IL-1β	CCCTAAACAGATGAAGTGCTCCT	CATGGCCACAACAACTGACG
IL-6	CAATGAGGAGACTTGCCTGGT	ATTTGTGGTTGGGTCAGGGG
IL-17A	TTCCCCCGGACTGTGATGGTCA	CAGGGTCCTCATTGCGGTGGAGA
TNF	GGCAGTCAGATCATCTTCTCGA	GGACCTGGGAGTAGATGAGGT
RANKL	AGAGAAAGCGATGGTGGATG	TATGGGAACCAGATGGGATG
OPG	CGCTCGTGTTTCTGGACAT	GTAGTGGTCAGGGCAAGGG
COL1A1	ACGAAGACATCCCACCAATC	AGATCACGTCATCGCACAAC
CTSK	CAGGGTCAGTGTGGTTCCTG	CCCCGGTTCTTCTGCACATA
OCL	CCAGGCAGGTGCGAAG	TCAGCCAACTCGTCACAGTC
TRAP	CAGTGGCCTCAGCGTTGAAT	CCCTGAGCCTTTATTCCCTCC
CBFA1	CGGAATGCCTCTGCTGTTA	TCTGTCTGTGCCTTCTGGGT
DKK1	CAGGCGTGCAAATCTGTCT	AATGATTTTGATCAGAAGACACACATA
SOST	AGACCAAAGACGTGTCCGAG	GGGATGCAGAGGAAGTC
AdipoQ	GGTGAGAAGGGTGAGAAAGA	TTTCACCGATGTCTCCCTTAG
AdipoR1	TTGTGTACAAGGTCTGGGAGG	GATGCTCTTGAAGCAAGCCC

IL – Interleukin; TNF – Tumor necrosis factor; RANKL - Receptor Activator of NF-kB Ligand; OPG – Osteoprotegerin; COL1A1 – Collagen type I; CTSK – Cathepsin K; OCL – Osteocalcin; TRAP – Tartrate resistant acid phosphatase; CBFA1 - Core-Binding Factor Alpha I; DKK1 - Dickkopf-related protein 1; SOST - Sclerostin; AdipoQ - Adiponectin; AdipoR1 - Adiponectin receptor 1.

DISCUSSION

The association between vascular and skeletal systems has been investigated over the last few years, particularly in the context of rheumatic inflammatory diseases where these systems are often altered in the same individual, such as in RA or SLE. In addition, the role of inflammation has also been studied in the development and progression of these diseases and of the comorbidities affecting the vascular and skeletal systems. The paradox between vascular calcification and low bone density has raised the curiosity of researchers, and several studies have been developed not only in mice but also in humans. However, the mechanisms underlying these processes have yet to be clarified.

The goal of the present work was to add knowledge to the processes underlying the interaction between vessels and bones in the context of rheumatic inflammatory diseases, specifically in RA and SLE, through the study of the effect of inflammation on the tissues.

The first part of this thesis focused on the effect of inflammatory rheumatic diseases on the vessels, leading to vascular alterations and CV disease. We have assessed the early vascular alterations observed in patients with RA and SLE, without previous CV events, through the determination of serum levels of vascular biomarkers and the study of endothelial function (part I). We found that patients had elevated serum levels of sICAM-1, thrombomodulin (TM) and tissue factor (TF) compared to controls. Also, vascular biomarkers correlated with inflammatory parameters and were found to be higher in active disease than in quiescent disease. Overall, early vascular changes were more pronounced in lupus. In SLE patients we found a marked increase in serum sICAM-1 and TM, whereas RA was characterized by elevated TF levels. Adhesion molecules might represent a link between inflammation and atherosclerosis, since their increased expression on atherosclerotic lesions has been previously documented [402,403] and the high serum levels of these molecules, in particular of sICAM-1, stand as an independent risk factor for atherosclerosis and a predictor of CV events [404,405]. Fibrinogen, when binding to ICAM-1, promotes adhesion and transendothelial migration of leukocytes, an initial step in vascular inflammation [51]. We have found, that not only fibrinogen was as an independent predictor of endothelial dysfunction in SLE and RA, but also both sICAM-1 and fibrinogen were higher in patients with SLE than in patients with RA. Expression of TM, a molecule with anti-coagulant properties, is decreased during the inflammatory process and elevated serum levels of this molecule are an indicator of probable endothelial injury [406]. Together with increased TF, an initiator of the extrinsic coagulation pathway, they create an environment favorable to thrombotic complications. We found no differences in endothelial function measured by PAT between patients and controls, nor between SLE and RA. However, patients with SLE had a higher AIx, which indicates a worse vascular condition.

The higher CV risk of lupus patients as compared to RA patients was corroborated by the more pronounced early vascular changes and more elevated vascular serum markers.

We have then assessed the incidence of CV events in the RA cohort at 5 years of follow up (part II), evaluating the contribution of traditional CV disease risk factors and RA-related parameters to future events. Four CV events occurred in this cohort, contributing to an incidence rate of 7 per 1000 person-years, which is much higher than what is estimated for the general Portuguese population without RA [407,408]. We have identified that ESR and sICAM-1 serum levels were independent predictors of the occurrence of CV events. A pro-inflammatory status has been associated with higher CV risk and mortality in RA patients [409], and it has also been shown that those with active disease have less stable carotid plaques [410]. The presence of carotid plaques is a helpful indicator of patients at higher risk of development of CV events. Also, the heterogenous type of plaques are more prone to evolve to plaque hemorrhage and thrombotic events [411–413]. In our study, those patients who suffered a CV event had mainly homogeneous plaques at baseline, unlike to what would be expected. However, it would be interesting to have a re-evaluation of these patients by carotid ultrasound to understand if some changes in the carotid plaques, together with other factors such as traditional CV risk factors, sICAM-1 levels and therapy, specifically with corticosteroids, might justify the evolution of those plaques to more vulnerable ones, leading to the occurrence of CV events.

We have further evaluated how bone metabolism biomarkers, specifically sRANKL and OPG, are affected by inflammatory rheumatic diseases, specifically in SLE patients (part III). We have found that OPG serum levels were lower in SLE patients than in controls and were inversely associated with anti-dsDNA levels, which in turn were directly related with disease activity defined according to SLEDAI-2K. Thus, those patients with a more active disease were more exposed to the effect of RANKL/RANK interaction as a consequence of diminished OPG levels. We have not found significant differences in serum RANKL levels between SLE patients and healthy controls, but the sRANKL/OPG ratio was increased in patients, at the cost of serum OPG decreased levels. Additionally, sRANKL/OPG ratio was found to be associated with anti-Sm antibodies, often present in active SLE [414–416]. An increased sRANKL/OPG ratio has been associated with bone loss in immune mediated inflammatory diseases [132,417] and it has been previously described in RA [418,419]. Our findings suggest that SLE per se has an important role in accelerating osteoclastogenesis, and consequently bone loss, since the sRANKL/OPG ratio was found to be independently associated with anti-Sm levels and was not associated with corticosteroids therapy.

At last, we aimed to understand the role of inflammation as a common contributor to the interplay between bones and vessels. For that we have first used a mouse model of atherosclerosis (ApoE$^{-/-}$) and evaluated both bones and vessels disturbances, as well as tested their association with inflammatory markers (part IV). Secondly, using human samples from organ donors and from endarterectomized patients with advanced atherosclerosis (part V), we have evaluated the role of inflammation in the relationship between bone metabolic disturbances, specifically OP, and vascular disease classifiable as atherosclerosis.

We have found that in ApoE$^{-/-}$ mice, comparing to wild type mice, the ratio between bone turnover markers CTX-I/P1NP increased as mice got older, as a result from both an increase in CTX-I serum levels and a decrease in P1NP serum levels, suggesting an activation of bone turnover over time. Also, histomorphometric analysis of these mice bones revealed a decrease in bone volume and trabecular thickness and an increase in trabecular separation, which were in accordance with an increased bone turnover over time. However, these results did not translate in increased bone fragility, as assessed by biomechanical testing. ApoE$^{-/-}$ mice have an accelerated progression of atherogenesis when fed a high-fat diet [388,420] and we have subjected both the experimental group and the control group to an atherogenic diet from 10 weeks old, to minimize the environmental differences between groups. However, there are some studies providing evidence that both age [392] and a high-fat diet [393,394] have an effect on ApoE$^{-/-}$ mice bone mass, leading to a decrease on bone formation, probably through blocking the differentiation of OB progenitor cells [395]. Thus, the lack of differences between ApoE$^{-/-}$ mice and the control group observed in our work might be justified by the diet. To confirm this hypothesis a comparison with animals fed a normal chow diet would be necessary.
We have further analyzed the expression of inflammation and bone remodeling proteins without finding any differences between groups neither over time, nor any relationship between the expression of these proteins and bone biomechanical or histomorphometric evaluations, reinforcing the idea that other factors, such as altered lipid transport and high-fat diet associated with mice aging, might play a more relevant role on atherosclerosis progression and bone disturbances than inflammation in this animal model.

In the last part of this project, using deceased donors' samples, we have found a positive correlation in the expression of several genes related to inflammation and bone remodeling between bones and vessels. Importantly, this is not a widespread finding, as confirmed by the lack of association with adipose tissue samples from the same individuals. Also, we found that

bone remodeling proteins were expressed on vessels of individuals without identified CV disease. Regarding bone remodeling proteins, we could expect an inverse relationship between bones and vessels, since an association between atherosclerotic lesions calcification and bone loss had been previously described [257,421,422]. However, we have found a direct relationship, leading us to hypothesize the existence of different patterns of expression in normality and in pathology, in line with the previous described [423] changes in the gene expression of calcification regulators in the setting of atherosclerosis progression. No effect of age or gender on gene expression pattern were found, pointing to an intrinsic connection between bone and vessel disturbances.

We have found that more than 45% of the patients with advanced atherosclerosis had low BMD, accordingly to the age range of this population and to previous studies [244,424]. In those patients with lower BMD, the expression of bone formation genes (*CBFA1* and *OCL*) on carotid atheroma plaques was lower, which could indicate a lower tendency to plaque calcification. However, no differences were found in the calcium content as determined by Alizarin Red S staining between patients with normal or low BMD. On the contrary, previous studies [425–427] have reported an association between low BMD and vascular calcification, suggesting that the analysis of the plaques at different stages of development might influence the genes expressed.

Statins were described as having a positive effect on bone metabolism [428], namely through an antiresorptive effect on bone cells, and inhibition of the inflammatory process [429], they have been simultaneously associated to an increase in carotid plaques calcification [430] aside to plaques regression and composition change [431–434]. Accordingly, increased gene expression levels of *OCL* (expressed by differentiated OBs) were found in the plaques of our patients under statins therapy. Nevertheless, we found that a high percentage (about 46%) of our patients under statins therapy have decreased BMD.

Lastly, carotid atheroma plaques immunohistochemical analysis revealed that both CD3 positive cells and CD68 positive cells were highly present in males' plaques, suggesting that plaques are more inflammatory in men than in women, as previously proposed [435,436]. Also, CD3 positive cells were infrequent in the plaques of patients with low BMD levels, which were expected to have echogenic (more calcified) plaques with lower inflammation [437–439], as calcification and inflammation are thought to be active at different stages of disease progression [440], with inflammation at early phases and calcification predominantly later [436,441,442].

CONCLUSIONS

Previous epidemiological studies have shown that CV diseases and osteoporosis are responsible for high rates of morbidity and mortality, share common risk factors, and are often present in the same individual, with the inflammatory process implicated in the pathogenesis of both diseases, particularly in patients with immune mediated rheumatic diseases.

In this PhD project we have confirmed the role of inflammation in the pathogenesis of atherosclerosis in chronic inflammatory rheumatic diseases. Moreover, baseline levels of inflammation and endothelial activation markers contributed significantly to the occurrence of CV events over the following 5 years. We also found differences between SLE and RA, with more pronounced early vascular changes in lupus patients.
We have also described a raised sRANKL/OPG ratio in female SLE patients, suggestive of increased osteoclastic stimuli in this group.
In the ApoE$^{-/-}$ mouse model, the altered lipid transport and a high-fat diet, associated with mice aging, may play a more relevant role than inflammation in the development of vessels and bone disturbances.
Additionally, in humans without inflammatory diseases we found a direct correlation of the expression of inflammatory and bone remodeling proteins between bones and vessels, independently of age and gender.

This work suggests that the association between atherosclerosis and osteoporosis rely on an intrinsic connection between the tissues involved and that the contribution of the risk factors for the progression of these diseases, namely inflammation, affect more pronouncedly patients with immune-mediated rheumatic diseases than healthy subjects. It is thus crucial to closely monitor bone quality in CV disease patients, as well as control atherosclerosis development in patients with poor bone quality to ensure early detection and prevention of the progression of these conditions. This strategy should be followed even more tightly in patients with immune mediated rheumatic diseases such as RA and SLE.

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